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Regulation of β2-integrins by signalling pathways and cytoplasmic interacting partners

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Declaration

I declare that I am the sole author of this thesis and all references have been consulted by me personally. The work, of which a thesis is a record, has been done by me, unless otherwise acknowledged. This work has not been previously submitted for a higher degree.

Hwee San Lek

Statement

I certify that Hwee San Lek has fulfilled the conditions of the University of Dundee and that she is qualified to submit the accompanying thesis in the application for the degree of Doctor of Philosophy.

Dr Susanna C. Fagerholm

Abstract

In the immune system, integrin-mediated adhesion is important for leukocyte trafficking, signalling, activation and effector functions, but how integrin-mediated adhesion is regulated in leukocytes is still incompletely understood. The main focus of this thesis was to investigate signalling pathways and mechanisms which regulate lymphocyte adhesion under flow conditions and integrin recycling and integrin-mediated phagocytosis in myeloid cells, respectively.

Traditionally, cell adhesion assays have been carried out in static conditions using immobilized ligands. We have now developed shear flow assays to study integrin-mediated cell adhesion and signalling pathways involved in a physiologically-relevant manner. Integrin regulation in naïve T cells has been studied extensively in the past, and therefore the focus of this thesis was to investigate signalling pathways that regulate β2-integrins (particularly LFA-1, Lymphocyte Function-associated Antigen 1) in B cells and effector T cells. It was now shown that B cells do not require Protein Kinase C βeta or Protein Kinase D for integrin-mediated cell adhesion but inhibition of Phosphoinositide 3-kinase and Akt reduced chemokine-(Stromal cell-Derived Factor 1-) induced B cell adhesion to the LFA-1 integrin ligand ICAM-1 (Intracellular Adhesion Molecule 1) under shear stress. In contrast, integrin-mediated adhesion of $CD4^+$ and CD8+ T cells was not affected by Akt inhibition under shear flow conditions, indicating that adhesion in different lymphocyte subtypes is regulated by different signalling pathways. I show here that effector cytotoxic T lymphocytes (CTLs) have high LFA-1 integrin expression and display high spontaneous binding to ICAM-1 under static conditions. Unlike B cells, these cells were able to adhere to ICAM-1 under shear stress in the absence of chemokines. However, cytotoxic T lymphocyte adhesion to ICAM-1 under shear flow was dependent on calcium/calmodulin signalling and an intact actin

cytoskeleton. β2-integrins in myeloid cells are continuously recycled and mediate both cell trafficking and phagocytosis of complement (iC3b)-coated particles, but how these processes are regulated remain incompletely understood. I show here that a triple threonine-motif in the β2-integrin cytoplasmic domain is important for in integrin-mediated adhesion in primary mouse macrophages and dendritic cells. This motif also prevents integrin lysosomal degradation in primary myeloid cells, and is required for integrin-mediated phagocytosis of iC3b-coated particles. I also show that an R77H substitution in the extracellular domain of the Mac-1 integrin, which is associated with the inflammatory disorder systemic lupus erythematosus, leads to impaired cell adhesion to ICAM-1 and iC3b as well as impaired phagocytosis.

In conclusion, in this thesis I have successfully developed methods to investigate integrin-mediated adhesion in leukocytes. In addition, I have investigated signalling pathways and mechanisms involved in regulation of β2-integrin-mediated functions in primary lymphocytes and myeloid cells. These studies have led to an increased understanding of how integrins in immune cells are regulated both in the presence and absence of shear stress.

1. Introduction and Review of Literature

1.1. Overview of the immune system

The immune system is responsible for protecting the body from disease, acting as a barrier against infections and maintaining a healthy balance during recovery from damage. The immune system has a complex organisation of various organs, cell types, proteins and signalling molecules. Barriers such as the skin and mucosal layer act as a physical barrier to block the entry of invasive pathogens. The first immune cells to react to invading pathogens are cells in the innate immune system, which provides generic defence function for the host without specificity to a particular pathogen. Cells of the innate immune system include neutrophils and other granulocytes, as well as phagocytes such as macrophages and dendritic cells that patrol the body to detect and quickly react to foreign particles and natural killer cells. These innate immune cells react quickly, engulfing bacteria, activating toll-like receptors, producing cytokines, and killing viral-infected cells by ligation of death receptors or release of cytolytic granules. The innate immune system also plays a crucial role in activating the adaptive immune system (Benoit et al., 2008, Bratton and Henson, 2011, Noris and Remuzzi, 2013).

The adaptive immune system is activated more slowly than the innate immune system and is made up of specialised cells that have specific targets but are highly adaptable. Cells in this system include subsets of B and T cells which are involved in humoral and cell-mediated immunity. Plasma B cells are involved in the production of antibodies that aid in the formation of immune complexes for clearance and aid cell activation. Membrane-bound forms of antibodies also serve as antigen receptors for B cells. Helper T cells that are activated by antigen presenting cells, such as dendritic cells, can activate

B cells and $CD8^+$ cytotoxic T cells, leading to antibody production and activated effector cytotoxic T cells can kill virus-infected cells. A pool of memory B and T cells persist in the immune system for long periods of time so that they can be activated quickly with enhanced capabilities during a new round of infection.

Natural killer cells which are considered part of the innate immune system can also be considered to be part of the adaptive immune system. Adoptive transfer of mature cytokine-stimulated Natural killer cells into naïve recipients led to a memory response. These cells were able to persist for 1 month in the new hosts and displayed enhanced cytokine production upon restimulation (Cooper et al., 2009).

The immune system is important in many aspects of human health and has to be kept in balance. The lack of ability to protect the body from infections and inflammation can lead to illness and even death. Therefore, deficiencies in innate immunity are rare and almost always lethal. Patients suffering from neutropenia display deficiency or low counts of neutrophils, which cause them to suffer from recurring bacterial and fungal infections (Boxer, 2012). As neutrophils also play a part in the regulation of both the innate and adaptive immune system, mice that lack neutrophils were found to induce hyper-responsive natural killer cells, another type of immune cell which is important for anti-viral and anti-tumour responses (Jaeger et al., 2012). Sufferers of primary immunodeficiencies have mutations in SCID genes which cause defects in the adaptive immune system. These mutations usually cause B cell defects, leading to lack of B cells and immunoglobulin production and also affecting T cell function. Patients usually suffer from severe recurrent and chronic infections such as bronchitis, pneumonia, gastrointestinal and ear infections from a young age until early adulthood (Felgentreff et al., 2011 Hoernes et al., 2011).

The immune system has to be tightly regulated so as not to over-react to self-antigens. A hypersensitive immune system has been implicated to be the cause of allergies and many autoimmune diseases such as multiple sclerosis, an autoimmune disease leading to damage in the central nervous system (Weissert, 2013) and rheumatoid arthritis, an inflammatory disease which affects the joints (Möttönen et al., 2005, Cooles et al., 2013). Systemic lupus erythematosus is a Type III hypersensitivity (antibody-antigen complex mediated) autoimmune disease often occurring in women of child bearing age. Lupus is characterised by flares and damage to multiple organs caused by a deficiency in immune-complex clearance. It is a multifactorial disease and both genetic risk factors as well as environmental stress triggers are involved in disease initiation (Liu and Davidson 2012, Lisnevskaia et al., 2013 Pieterse and van der Vlag, 2014).

1.2. Cells in the Immune system

Cells in the innate immune system include granulocytes (such as neutrophils, eosinophils and basophils/mast cells), as well as macrophages, dendritic cells and natural killer cells, among others (Table 1). Phagocytes such as neutrophils, macrophages and dendritic cells have the ability to phagocytose pathogens and are important in innate immunity.

Immune cell type				Functions	
Granulocytes	Neutrophils			Innate Immunity	Phagocytosis, Reactive Oxygen Species production, neutrophil extracellular traps production
Myeloid cells	Macrophages				Professional phagocytes. M1: Inflammatory response, M2: suppressive response
	Dendritic cells				Phagocytosis, antigen presentation. Plasmacytoid DCs: circulatory. Classical DCs: reside in lymphoid and non-lymphoid tissues.
	Monocytes				Circulating cells that could differentiate into macrophages or dendritic cells
Lymphocytes	Bone Marrow Maturation	Natural Killer cells		Innate and Adaptive Immunity	Cytokine production to reject tumours and regulate dendritic cell, B/T cells activation, kill viral-infected cells
		Mature B cells		Adaptive Immunity	Cytokine production, regulation of cell survival, presentation to Helper T cells
		Plasma B cells			Antibodies production
		Memory B cells			Persist in hosts bone marrow. Stimulation leads to quick differentiation into plasma B cells for faster antibodies production
	Thymus Maturation	CD4+	T_H1		Production of interferon-gamma, TNF-α
			T_H2		Production of IL-2, IL-4, IL-5, IL-13, IL-9, TNF-α
			T_H17		Production of IL-17A, IL-17F, IL-22, IL-21, TNF-α
			Cytotoxic CD4+ T cells		Weak cytotoxic cells but aid killing tumour/viral-infected cells
			Regulatory T cells		Suppressor cells maintaining immune homeostasis, regulate Helper T cells cytokine production. Self tolerance
		CD8+	Cytotoxic T cells		Killing of infected cells by release of cytolytic granules or ligation of death receptors
			Memory T cells		T_{CM}: central memory T cells homing to lymphoid organs with ability to proliferate quickly when stimulated. T_{EF}: able to activate effector functions quickly when stimulated
		Natural Killer T cells		Innate and Adaptive Immunity	Attacks solid tumour by lysing tumour cells, activate Natural Killer and CD8+ cells
		γδ T cells			Innate-like cells that influence host responses. Resides in skin, liver and mucousal lining of digestive, reproductive and reproduction organs

Table 1: Types of immune cell types and summary of their function.

Neutrophils are also known as polymorphonuclear cells (PMNs) due to their characteristic multi-lobed nucleus. These cells are rapidly recruited to sites of infection via extravasation out of blood vessels using surface adhesion receptors such as PSGL-1 and β2-integrins (Amulic et al., 2012). These cells originate from the bone marrow and their production is controlled by G-CSF (granulocyte colony stimulating factor). 50-70 % of leukocytes in the circulation in humans are neutrophils, in contrast to only 10-20 % in mice (Kolaczkowska and Kubes, 2013). Neutrophils are short lived and have a life span of 6-8hours in the circulation in humans. They are mainly cleared by macrophages or by dendritic cells in situ after apoptosis, or drain to lymph nodes from the airways, gut or gingival crevices (Kobayashi et al., 2005, Bratton and Henson, 2011, Kolaczkowska and Kubes, 2013).

Neutrophils have pattern recognition receptors such as Toll-like receptors on their surface that can recognise surface bound or secreted molecules such as bacteria-derived peptidioglycan, lipoproteins, lipopolysaccharide, CpG-containing DNA and flagellin known as PAMPS (pathogen-associated molecular patterns). The infectious pathogens can be ingested by neutrophils through a process known as phagocytosis, which can be aided by opsonisation (coating of particles) by, for example, the complement system or by antibodies (Kobayashi et al., 2005). After phagocytosis, the production of reactive oxygen species is triggered, and the phagocytosed bacteria are killed by fusion with cytoplasmic granules which contain antibacterial proteins such as cathepsins, defensins, lactoferrin and lysozyme. NETs (neutrophil extracellular traps) consist of DNA and other granular proteins that are released by highly activated neutrophils and these can trap pathogens to further facilitate clearance (Kolaczkowska and Kubes, 2013). Defects in neutrophil function can lead to inability to fight infections observed in leukocyte adhesion deficiency patients (characterised by reduced or lost expression or function of

adhesion receptors) or unresolved inflammation observed in Rheumatoid Arthritis (Alon et al., 2003, Amulic et al., 2012).

Macrophages are professional phagocytes which scavenge and ingest pathogens, dead tissue and debris. There are different types of macrophages with distinct immune functions, such as M1 (classically activated, inflammatory) type macrophages which mediate host defence to bacterial, protozoal and viral infection and display anti-tumour immune responses. M2 (alternatively activated, suppressive) macrophages have anti-inflammatory functions and regulate wound healing. Regulatory macrophages secrete anti-inflammatory cytokines and tumour-associated macrophages suppress anti-tumour immunity. Macrophages can also be sub-classified according to their location, such as osteoclasts in bones, microglial cells in the brain, eyes and testes, alveolar macrophages in lungs, histiocytes in interstitial connective tissue and Kupffer cells in the liver. During infection, the chemokine CCL2 secreted by inflamed tissues and fibroblasts encourage monocyte extravasation from bone marrow and spleen and these cells differentiate into macrophages in tissues. Macrophages phagocytose infectious agents and activate helper T cells to produce interferon gamma that drives the M1 response (Murray and Wynn, 2011).

Other notable cells in the immune system are dendritic cells, which are the most important antigen presenting cells (APC) of the immune system. Dendritic cells capture and present antigens from pathogens to B or T lymphocytes. There are 2 major groups of dendritic cells: plasmacytoid dendritic cells (which accumulate in the blood and lymphoid tissues and enter the lymph nodes through the blood circulation) and classical dendritic cells (a small subset of tissue hematopoietic cells that populate most lymphoid and nonlymphoid tissues, which have an enhanced ability to sense tissue injuries and

process and present phagocytosed antigens to T cells to prime naïve T cell responses). Further subsets of dendritic cells include non-lymphoid tissue classical DCs, lymphoid organ-resident DCs, tissue migratory DCs and Langerhans dendritic cells that are found in the epidermal layer of the skin (Merad et al., 2013). Monocytes from the blood, bone marrow and spleen can also differentiate into macrophages and dendritic cells during inflammation (Geissmann et al., 2010). Natural killer cells have cytotoxic and cytokine production capabilities, and are involved in the killing of virus-infected cells, rejection of tumours and regulation of dendritic cells, B and T cells. They are found in all tissues but as a minor fraction in both mice and humans with a life span of 2 weeks (Vivier et al., 2008).

Lymphocytes are white blood cells in the immune system that play a central role in adaptive immunity. There are two main types of lymphocytes, T cells and B cells, which have different receptors on their cell surfaces and are involved in different functions (Table 1). B cells are important in humoral immunity (antibody responses) while T cells are involved in cell-mediated immunity. There are many types of T cells such as helper T cells and cytotoxic T cells, with different roles in immunity.

During T cell activation, antigen-presenting cells make contact with T cells and this contact requires adhesion molecules such as LFA-1 and ICAM-1 in addition to the T cell receptor on the T cell surface and antigen-binding MHC molecules on the antigen presenting cell. These contacts are necessary for co-stimulation of naïve T cells (Pribila et al., 2004, Wang et al., 2009). Naïve $CD4^+$ T cells can differentiate into Helper T cells known as: T_H1, T_H2 and T_H17. T_H1 cells produce high amounts of interferon-gamma, IL-2 and exclusively produce lymphotoxin while T_H2 cells do not produce interferon-gamma or lymphotoxin but do produce some IL-2. In addition, T_H2 cells produce IL-4,

IL-5, IL-13 and sometimes IL-9. Both cell types produce TNF-α. T_H17 cells are characterized by the production of IL-17A, IL-17F, and IL-22 as signature cytokines and are good producers of IL-21 (Zhu et al., 2010).

Regulatory T cells are suppressor cells that generally arise from naïve $CD4^+$ T cells. These cells are important in the regulation of immune homeostasis and in maintenance of self-tolerance. Foxp3 has been identified as an important transcription factor that regulates the lineage of regulatory T cells, and deficiency of Foxp3 results in lack of regulatory T cells, leading to lethal autoimmunity. Regulatory T cells are also important during differentiation of T cells and affect production of cytokines by Helper T cells (Corthay 2009, Josefowicz et al., 2012).

Naïve $CD8^+$ T cells can differentiate to become effector cytotoxic $CD8^+$ T cells (also known as CTLs or effector CTLs) which kill infected cell quickly by releasing cytolytic granules, which contains molecules such as FasL and perforin, or by ligation of death receptors at the immunological synapse. Most $CD4^+$ T cells have weaker cytolytic abilities and are instead involved in the production of cytokines as Helper T cells. However, the ability of $CD4^+$ cytotoxic T cells to recognise MHC Class II may be of additional help in the killing virally-infected or tumour cells (Jenkin and Griffiths, 2010, Marshall and Swain, 2011).

Natural Killer T cells are a subset of T cells that have the characteristics of both natural killer cells and T cells. These cells make up about half the population of T cells in the liver and have an important role in fighting solid tumours by lysing tumour cells directly or through the interferon gamma-mediated activation of natural killer cells and $CD8^+$ T cells (Ghalamfarsa et al., 2013). Memory T lymphocytes persists for life and

can be classified into central memory (T_{CM}) and effector memory (T_{EM}) cells due to their distinct homing capacity and effector functions. Effector memory T cells migrate to tissues and are able to confer immediate effector responses while Central memory T cells home to secondary lymphoid organs, have no effector functions but can be quickly stimulated to proliferate and differentiate (Sallusto et al., 2004). Gamma delta T cells (γδT cells) are innate-like cells that can influence the host response to injury, infections and malignancy and are localized to specific anatomical sites such as skin, liver and the mucosal layers of digestive, respiratory or reproductive organs (Bonneville et al., 2010).

B cells are produced in the bone marrow and then migrate to the spleen and lymph nodes where they mature. Mature B cells have antibodies as their surface receptors. B cell receptors bind to foreign antigen, leading to B cell activation. This will trigger the production of more antibodies by plasma B cells. Antibodies are required for various immune-mediated responses such as the recruitment of complement for cell killing and opsonisation of pathogens for phagocytosis (Tobón et al., 2013). B cells have regulatory functions such as promoting cell survival, chemokine and cytokine production and interacting with T helper cells, which help fight bacterial and viral infection and also cancer (Batista and Harwood, 2009).

Memory B cells are cells that were activated in a primary response but do not have an effector function. The primary B cell response peaks at 4 weeks in human. These cells persist in the host for more than a year after the resolution of the response without proliferation in the bone marrow so that the future response and stimulation can lead to the differentiation of these cells into memory plasma cells. This enables faster and enhanced production of specific antibodies, isotype switching and affinity maturation. Memory B cells have been detected after more than 50 years after smallpox vaccination

and the specific serum antibody titers have half-lives between 50–200 years (Tangye and tarlington, 2009, Yoshida et al., 2010).

Lymphocyte development involves various stages of cell proliferation, differentiation and activation, which gives rise to subsets of B and T cells and development of self-tolerance in the adaptive immune system. Early progenitor cells are found in the bone marrow, migrate to spleen (B cells) and thymus (T cells) for the maturation progress, are released into the circulation, and then migrate to secondary lymphoid organs and other sites to mediate their effector functions. These processes require cell migration within tissues and interactions with other cells in the immune system. Lymphocytes also need to overcome shear stresses when leaving the bloodstream to get to other sites. Lymphocytes therefore require tight regulation of adhesion molecules to control migration processes related to cell differentiation and for their effector functions.

In the bone marrow, B cell precursors first rearrange their immunoglobulin genes and undergo differentiation to become immature B cells. After negative selection, immature B cells leave the bone marrow and enter the circulation and home to the spleen to develop into mature B cells (Kantor et al., 1993). In the spleen, immature B cells develop into mature marginal zone B cells (MZ) or mature follicular B cells. These processes are regulated by Notch signalling and NFκB signalling downstream from the B cell receptor (Saito et al., 2003). Marginal zone B cells do not recirculate and reside at the border between white and red pulp of the spleen due to high expression of LFA-1 and VLA-4 (Okada and Cyster, 2006). These cells respond to blood-borne antigens without T cells. Immature cells differentiate into transitional 1, transitional 2 and then follicular B cells when entering the white pulp of spleen and respond to T cell-dependent antigens (Okada and Cyster, 2006). Some mature follicular B cells leave the

spleen, enter into the circulation and then enter lymph nodes and mucosal-associated lymphoid tissues. Follicular B cells migrate within the lymph nodes to follicles where they come into contact with antigen. B cells that meet antigen in spleen and lymph nodes then differentiate into plasma cells or memory B cells (MacLennan et al., 1997).

During T cell development, progenitor cells from bone marrow enter the thymus for maturation. These cells are still naïve as they have not encountered antigen. Mature T cells that mainly express either $CD8^+$ or $CD4^+$ leave the thymus and enter lymph nodes, mucosal lymphoid organs and spleen (Anderson et al., 2006). Mature but naïve T cells express CD62L (L-selectin, an adhesion molecule necessary for adhesion to high endothelial venules), CCR7 (a homing receptor for T cells) and LFA-1 integrins (which are required for firm adhesion to endothelium and also facilitates T cell activation by antigen-presenting cells). Antigen presenting cells that have picked up antigens from peripheral tissues will meet T cells in secondary lymphoid tissues and presented antigens via MHC Class I and Class II receptors to $CD8^+$ and $CD4^+$ T cells, respectively. $CD8^+$ and $CD4^+$ effector T cells then migrate to peripheral tissues. T cells that express $CD62L^-CXCR5^+CD4^+$ migrate to B cell follicles to activate B cells to become plasma B cells and produce antibodies, while CCR7-CD62L-CD8+ T cells travel to non-lymphoid tissues such as lungs and gut to clear pathogens (Broere et al., 2011). Memory cells that express CCR7 can recirculate through lymphoid tissues (Anderson et al., 2006). Recirculation of T cells occurs until these cells meet antigen or die of neglect. T cells can be retained in the spleen for a few hours and up to a day in lymph nodes and leave via splenic veins and efferent lymphatics (Broere et al., 2011).

1.3. Overview of integrin functions in the immune system

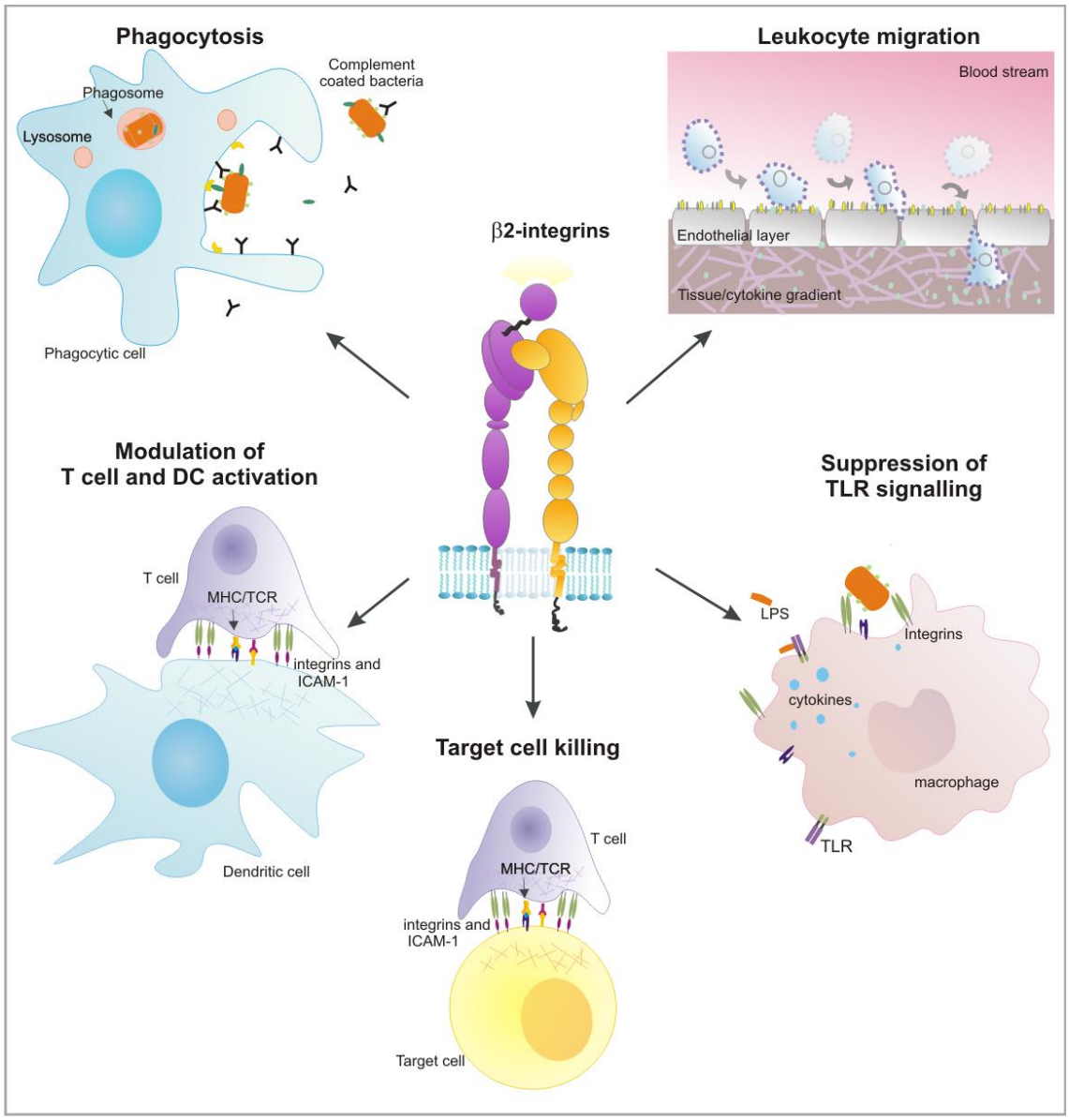


Figure 1. Schematic diagram showcasing some of the important functions in the immune system that require integrins.

The β2-integrins are specific to leukocytes. They regulate various processes in the immune system such as extravasation, signalling, cell-cell interaction and phagocytosis.

Integrins, together with selectins are necessary in the process of leukocyte extravasation. Selectins slow down the leukocytes in the blood stream causing the cells to roll on the endothelial cell layer while activated integrins stop the cells and allow adhered cells to squeeze through the endothelium (Worthylake and Burridge, 2001, Hogg et al., 2003). Integrins are also needed for activation of T cells and dendritic cells (Burbach et al., 2007). It has also been shown that integrins are necessary for mediating NK (Natural killer) cell and neutrophil killing (Spriel et al., 2001, Weitz-Schmidt et al., 2009). In addition, integrins play an important part in integrin-mediated phagocytosis. Complement coated bacteria are taken up by Mac-1 integrin-rich cells such as dendritic cells and macrophages and digested during immune clearance (Fagerholm et al., 2006, Dupuy and Caron et al., 2008, Oliva at al., 2008) (Figure 1, MacPherson et al., 2013). Integrins are also required to dampen TLR signalling in macrophages (Yee and Hamerman 2013).

1.4. Classes of Integrins in the immune system

Integrins are obligate heterodimeric transmembrane receptors that mediate cell-cell or cell-matrix adhesion and migration. The integrin subunits consist of a short cytoplasmic tail, a transmembrane region and a large extracellular domain. 18 α subunits and 8 β subunits have been identified at present. These can be assembled into 24 different heterodimers in vertebrates. β subunits are generally present in excess but the number of integrin receptors on the cell surface is dependent on the amount of α subunits available. There are no free α and β subunits on the cell surface. The subunits determine ligand specificity (Barczyk et al., 2010). In leukocytes, the major types of integrins expressed are the β2 type (Table 2). These are also known as CD18 family integrins. The β2

subunit is paired with αL, αM, αX or αD subunits. αLβ2 (LFA-1) is also known as CD11a/CD18. αMβ2 (Mac1) integrin or CD11b/CD18 is important for integrin mediated phagocytosis and is abundant in phagocytic cells such as macrophages and neutrophils. Mice lacking β2 integrins suffer from chronic dermatitis and skin ulcerations, enlarged spleens, high neutrophil counts and high levels of immunoglobulins and plasma cells. T cell proliferation was also affected in these mice (Scharffetter-Kochanek et al., 1998). It was also found that there are reduced B and T cells in inguinal and axillary lymph nodes but a significant increase in CD18-/- TCRαβ double-negative T cells accumulating in cervical lymph nodes, which were poor in trafficking, lack regulatory functions but had intermediate TCR expression levels, behaving like NK cells and were thought to compensate for poor adaptive immunity in these mice (Oreshkova et al., 2009). CD18-/- neutrophils were found to utilize E-selectins and P-selectins for cell adhesion and rolling and were unable to emigrate to the peritoneal cavity (Walzog et al., 1998, Forlow et al., 2002).

Integrin subunits	**Expression**
αLβ2 (CD11a, CD18)	All leukocytes
αMβ2 (CD11b, CD18)	Monocytes, macrophages, NK cells. neutrophils and γδ T-cells
αXβ2 (CD11c, CD18)	Monocytes, macrophages, dendritic cells and NK cells
αDβ2 (CD11d, CD18)	Macrophages and eosinophils

Table 2. Members of β2 integrins and their expression in leukocyte populations.

Table modified from Tan, 2012, Bioscience Reports

β1 integrins, which are also known as fibronectin receptors or collagen-binding receptors are expressed in most cell types, including leukocytes, while β7 integrins are homing receptors on leukocytes which bind mucosal addresin cell adhesion molecule-1 (MAdCAM-1) (Gahmberg et al., 2009). Homozygous deletion of β1 integrins is embryonically lethal and required for morphogenesis of the inner cell mass (Stephens et al., 1995). Conditional knock-out mice lacking β1 integrins or expressing inactive β1 integrins had prolonged bleeding times due to insufficient activation of Rac-1, actin dynamics, granule secretion, and platelet aggregation (Petzold et al., 2013).

1.4.1. VLA-4

VLA-4 (Very Late Antigen-4) is also known as the α4β1 integrin and binds to ligands such as fibronectin, osteopontin thrombospondin, MadCAM-1 and VCAM-1 (Humphries et al., 2006). VLA-4 (α4β1) binds readily to fibronectin in the extracellular matrix and allows the adhered cell to spread and migrate (Elices et al., 1990, Alon et al., 1995). Patients suffering from rheumatoid arthritis have increased amounts of fibronectin secreted into the synovial fluid of rheumatoid joints, which causies the formation of a fibronectin gradient which facilitates influx of T cells expressing VLA-4 (Laffón et al., 1991).

VLA-4 binds to VCAM-1 (Vascular cell adhesion molecule-1) to mediate lymphocyte tethering, rolling and cell arrest under shear stress and the receptor is thought to be important for lymphocyte extravasation (Alon et al., 1995, Hyun Write et al., 2009). VCAM-1 is abundantly expressed in lung bronchioles. VLA-4, but not LFA-1 is important for the recruitment of neutrophils and T cells into lungs during *Streptococcus*

pneumoniae infection. Neutrophils depend on both Mac-1 and VLA-4 for recruitment from the circulation into the lungs (Kadioglu et al., 2011). In T cells, VLA-4 is accumulated in the lamellipodia and is activated by Rap1 (Hyun et al., 2009). VLA-4 is also needed for homing of CD34+ haemopoetic stem cells to bone marrow and spleen and for repopulation of CD34+ cells in the bone marrow (Lapidot et al., 2005).

VLA-4 is also important in the formation of immune synapse in the activation of T cell by a B cell or dendritic cell acting as the antigen-presenting cell. VLA-4 co-localises with LFA-1 at the peripheral supramolecular activation complex (pSMAC) of the immune synapse, which is the ring that surrounds the T cell receptor and MHC complex to strengthen the interaction. In this case, VLA-4 has an important regulatory role in providing co-stimulatory signals for T cell activation. In the presence of α4 antibodies, VLA-4 co-localised with CD3ζ (associated with the T cell receptor) and promotes Helper T cell response, leading to differentiation of CD4+ T cells and naïve T cell priming by dendritic cells (Mittelbrunn et al., 2004).

1.4.2. LFA-1

LFA-1 (Leukocyte Function-associated Antigen-1) or αLβ2-integrin binds to ICAM-1 and ICAM-2 ligands present on endothelial cells. These interactions mediate the firm adhesion between the leukocyte and the endothelial cells under conditions of shear flow (Issekutz et al., 1999, Shamri et al., 2005, Steiner et al., 2010. Gorina et al., 2013). Also, the formation of the immunological synapse between, for example, an antigen-presenting cell and a T cell is dependent on LFA-1 and ICAM-1,-2 or -3, and is necessary for antigen presentation, T cell activation and tolerance. LFA-1 can also bind

to ICAM-4 and ICAM-5 (Saloman and Blueston, 1998, Bleijs et al., 2000, Montoya et al., 2002, Carrasco et al., 2004, Švajger et al., 2010, Zumwalde et al., 2013).

LFA-1 integrins on B cell promote cell survival by binding to ligands on follicular dendritic cells and macrophages (Arana et al., 2008). CD11a was found to be necessary for T_H2 cell homing but not for T_H1 cell homing, which uses the CD29/β1 integrin. T_H2 cells lacking CD11a could still differentiate and be activated but displayed a reduced allergic response in the lung and in response to Leishmania infection in mice (Lee et al., 2008). T_H1 responses are responsible for delayed type hypersensitivity response, autoimmune disease and rejection of allografts while overactive T_H2 response have a role in parasitic infections and allergic responses (Ozdemir et al., 2009). Lack of CD11a in mice also affected the $CD8^+$ cell response and differentiation (Bose et al., 2013). CD11a mutations associated with increased integrin adhesion in mice were reported to lead to enhanced T_H2 cells accumulation in vivo in response to *Leishmania major* and *Aspergillus niger* exposure. Patients with CD11a polymorphisms were reported to suffer from atopic dermatitis and allergic rhinitis, associated with high eosinophil counts and serum IgE levels (Knight et al., 2014). On the other hand, obese mice displayed upregulation of LFA-1 and accumulation, increased activation and proliferation of $CD8^+$ T cells in adipose tissues (Jiang et al., 2014). These findings emphasize the importance of LFA-1 in regulating the immune response, aside from being an adhesive molecule.

1.4.3. Mac-1

Mac-1 (also known as Macrophage-1 integrin or Complement Receptor 3) or αMβ2-integrin is abundantly expressed on phagocytic cell types such as macrophages and dendritic cells and is important both for nonopsonic (Type I) and opsonic (Type II) phagocytosis (Cabec et al., 2002). Mac-1 binds to ligands such as iC3b for integrin-mediated phagocytosis (Plow et al., 2000) and to ICAM-1 and ICAM-2 to mediate crawling on endothelial cell layers during extravasation (Sumagin et al., 2010). Mac-1 also has many other ligands, such as fibrinogen (a coagulagent factor), heparin/glucosaminoglycan, LDL-receptor related protein, glycoprotein Ib on platelets, factor X, Matrix metallopeptidase-9, RAGE and JAM-C for paracellular migration between endothelial cell layers (Diamond et al., 1995, Plescia and Altieri, 1996, Simon et al., 2000, Spijkers et al., 2005, Fagerholm et al., 2006, Hirahashi et al., 2006 Lange-Sperandio et al., 2006, Kaneider et al., 2010).

Type I phagocytosis, mediated by the immunoglobulin receptor, is mediated by Cdc42 and Rac signalling, and type II, mediated by the complement receptor, is mediated by Rho, which controls actin remodelling (Caron and Hall 1998). Dectin-1, a major β-glucan receptor on macrophages can activate Mac-1 signalling to recognise fungal components and to recruit neutrophils for clearance. These processes require Vav-1 and Vav-3 RhoGTPase signalling (Lee et al., 2011).

In addition to its role in phagocytosis, Mac-1 also plays a role in leukocyte crawling under shear flow conditions in neutrophils (Ding et al., 1999, Simon and Goldsmith et al., 2002). Phosphorylation of the αM chain is necessary for integrin activation and

binding to ICAM-1 and ICAM-2 and for cell extravasation from blood vessels, but not for iC3b-mediated phagocytosis (Fagerholm et al., 2006).

Mutations in the Mac-1 α-chain, such as rs1143679 (R77H) in the ITGAM/αM integrin chain, are associated with SLE development, possibly through effects on immune complex clearance (Harley et al., 2008, Moser et al., 2009, Ding et al., 2013, Fossati-Jimack et al., 2013). Patients with SLE suffered from the effects of impaired clearance of apoptotic cells by macrophages (Gresham et al., 1991, Gaipl et al., 2005, Hepburn et al., 2006, Goldblatt et al., 2009). Mac-1 is also important for cell signalling: The same mutations in Mac-1 that were suggested to affect phagocytosis in SLE patients also contribute to dysregulation of B cell tolerance as Mac-1 negatively regulates B cell signalling (Rhodes et al., 2005, Ding et al., 2013). Mac-1 has also been suggested to be able to recognise extracellular dsDNA to enhance TLR3-dependent signalling in macrophages (Zhou et al., 2012). Although both LFA-1 and Mac-1 are integrins involved in adhesion and cell signalling, cells utilizing LFA-1 migrated over longer distances while Mac-1 is more important for cell migration during inflammation and has an important role in phagocytosis (Sumagin et al., 2010).

1.4.4. CD11c/CD18 integrin

CD11c/CD18 is made up of the αX-chain pairing with β2 and has 63% homology with CD11b (Frick et al., 2005). CD11c is mainly expressed on dendritic cells, monocytes, natural killer cells, myeloid cells, neutrophils, and can bind to ICAM-1, ICAM-2, VCAM-1, (Sadhu et al., 2007) and fibrinogen (Loike et al., 1991, Singh-Jasuja et al., 2013). CD11c/CD18 is also known as CR4, and binds to iC3b (like Mac-1) to mediate

phagocytosis of complement coated particles. Although CD11c is expressed at lower levels than CD11b on neutrophils, neutrophils depend on CD11c to bind to fibrinogen (Sadhu et al., 2007). CD11c is often used as a marker for mouse dendritic cells. Plamacytoid dendritic cells expressed low levels of CD11c while myeloid dendritic cells express high levels of CD11c. However, CD11c on bone marrow-derived dendritic cells and dendritic cells from spleen can be downregulated after activation by Toll-like receptors 3/4/9 (Singh-Jasuga et. al., 2013). CD11c is also expressed by mouse macrophages, granulocytes and T cells (Hume, 2008). CD11c is also involved in the regulation of cytokine production. Macrophages that express CD11c have high levels of pro-inflammatory cytokines and no detectable IL-10 while CD11c null cells produces high levels of anti-inflammatory IL-10 (Patsouris et al., 2008).

1.4.5. CD11d/CD18 integrin

CD11d is expressed on myeloid cells such as macrophage foam cells, splenic red pulp macrophages and neutrophils (Noti, 2002). Expression of CD11d is low in peripheral blood leukocytes but can be upregulated by chemokine stimulation (Van der Vieren et al., 1995). CD11d/CD18 is a multiligand receptor and binds to ligands similar to those recognised by Mac-1, such as fibronectin, vitronectin and fibrinogen (Yakubenko et al., 2006). CD11d appears to be important for migration of monocytes and accumulation of macrophages in the peritoneum. When present at low density, CD11d cooperates with β1- and β3-integrins during cell migration, while at higher density, CD11c inhibits β1- and β3-integrin mediated cell migration (Yakubenko et al., 2008).

The role of CD11d appears to be important in inflammation. Expression of CD11d is high in macrophage foam cells within atheroscleric plaques (Noti, 2002). CD11d plays a role in activation of macrophages, neutrophils, eosinophils (Yakubenko et al., 2008), in T cell proliferation (Wu et al., 2004), and infiltration of leukocytes into inflamed tissues (Mabon et al., 2000), lung inflammation (Shanley et al., 1998) and inflammatory response to spinal cord injury (Gris et al., 2004, Bao et al., 2011). Neutrophils, monocytes and macrophages infiltrate site of spinal cord injury to clear the area of bacterial infection, cellular debris and apoptotic cells. Pro-inflammatory cytokines are also produced by phagocytes. These contribute to secondary damage and can be reduced using CD11d monoclonal antibodies which blocks extravasation of neutrophils and macrophages (Mckillop et al., 2009, Saville et al., 2004). CD11d is also significantly upregulated in white adipose tissues of obese rodents and woman. Obesity lead to increase white adipose tissue expression of CD11b and insulin resistance, tissue inflammation and macrophage infiltration (Thomas et al., 2011).

1.5. Integrin ligands

Integrins bind to multiple different ligands. The ligand and integrin binding pairs can be classified into 4 main classes: RGD-binding integrins, LDV-binding integrins, A-domain containing β1 integrins and Non-αA-domain-containing laminin-binding integrins (Humphries et al., 2006).

The first class of ligand and integrin pair is the RGD-binding integrins, consisting of all five αV integrins, two β1 integrins (α5, α8) and αIIbβ3 which can recognise ligands containing an RGD tripeptide active site, such as thrombospondin, fibronectin,

fibrinogen and vitronectin. The second class, LDV-binding integrins (β2 integrins, some β1 and β7 integrins) bind to the acidic motif known as LDV which is related to RGD. Some of these ligands include fibronectin, VCAM-1 and MAdCAM-1. Other similar motifs in ligands in this group are collectively called the L/I-D/E-V/S/T-P/S. The LDV peptide motif is SVVYGL through which osteopontin interacts with α4β1, α4β7 and α9β1integrins. The β2-integrin family binds to ligands through an inserted A-domain in the α subunit and the binding is similar to LDV-binding, except that β1/β7-integrins use aspartate while β2 integrins use glumatate for cation coordination. Such ligands include, ICAMs, E-cadherin, iC3b, factor X and fibrinogen. The last two classes of ligand and integrin binding pairs are: A-domain β1 integrins bind distinctly to laminin, collagen or thrombospondin while other β1 integrins and α6β4 in the non-αA-domain-containing integrins only bind to laminin and thrombospondin (Humphries et al., 2006).

Common ligands of β2-integrins include ICAMs - intercellular adhesion molecules of the immunoglobulin superfamily. ICAMs are transmembrane glycoproteins that has immunoglobulin-like domains and are also found as soluble proteins in plasma. ICAM-1 (CD54) is the major ligand expressed on leukocytes, endothelial cells and epithelial cells in organs and has 5 immunoglubolin-like domains (Gahmberg et al., 2009). ICAM-1 is generally weakly expressed in resting conditions but can easily be induced by cytokines such as TNF-α (Tumour necrosis factor alpha) or LPS (lipopolysaccharide) at sites of inflammation (Arkin et al., 1991). Shear stresses can also upregulate the expression of ICAM-1 and E-selectin on human endothelial cells (Nagel et al., 1994). ICAM-2 (CD102) exists as a monomer with 2 immunoglobulin-like domains on leukocytes and on endothelial cells, providing co-stimulatory signals for T cells during aggregation, and also functions in natural killer cell (NK) cytotoxicity and the migration

of NK cells. However, the expression of ICAM-2 is not as easily up-regulated by proinflammatory signals (Gahmberg et al., 2009).

LFA-1 binding to ICAM-1 was shown to be stronger than binding to ICAM-2 using atomic force microscopy (Wojcikiewicz et al., 2006). Blocking of both ICAM-1 and ICAM-2 affected recirculation of circulating T cells through lymph nodes but ICAM-1 was necessary for migration of T cells to sites of inflammation and trapping in lungs (Lehmann et al., 2003). For arrest in high endothelial venules in the presense of shear stress, both ICAM-1 and ICAM-2 are needed initial cell arrest. Blocking of ICAM-1 blocks initial cell arrest in early stages but cells can overcome this by using ICAM-2 later (Boscaci et al., 2010). Lymphocyte crawling on high endothelial venules after adhesion is dependent on ICAM-1 (Boscaci et al., 2010, Soriano et al., 2011). Perivascular trapping which occurs when cells try to get through the basement membrane underlying high endothelial venules and fibroblastic stromal cells required ICAM-1 or ICAM-2. B cells and T cells are dependent on ICAM-1 but not ICAM-2 for interstitial motility (Boscaci et al., 2010).

ICAM-3 is expressed on most leukocytes and has a role in stabilizing T cell-Antigen presenting cell interactions (Berney et al., 1999). The structure of ICAM-3 is similar to ICAM-1 with 5 immunoglobulin-like structures and exists as a dimer (Ghamberg et al., 2009). LFA-1 on resting T cells bind poorly to ICAM-3 but ICAM-3 is able to boost LFA-1-mediated adhesion to ICAM-1 by encouraging clustering of LFA-1 integrins on the cell surface, leading to strong adhesion necessary for T cell proliferation (Bleijs et al., 2000). ICAM-4 is specifically expressed on red cells and is a ligand for LFA-1, Mac-1 and CD11c/CD18 ($\alpha X\beta 2$-integrin), which exists as 2 immunologlubulin-like structures and exists as a monomer (Ghamberg et al., 2009). The interaction of ICAM-4

and β2 integrins has been suggested to be important for the removal of red blood cells from the circulation by macrophages in the spleen during red blood cell development and turnover (Ihanus et al., 2007). The fact that ICAM-4 can also bind to the platelet integrin $\alpha_{IIb}\beta_3$ suggested that this interaction may be involved in platelet-erythrocyte aggregate formation during coagulation (Hermand et al., 2002). ICAM-5 is also known as telencephalin and is only expressed on telencephalic neurons of the central nervous system and soma-dendritic regions of neurons in the mammalian brain and not in immune cells (Yang, 2012, Tian et al., 2008). It can exist in dimers or tetramers and has 9 immunoglubulin-like domains (Gahmnberg et al., 2009). ICAM-5 acts as an anti-inflammatory agent and the soluble form, shedded from neurons, can be detected in physiologic fluids during brain ischemia, epilepsy, and encephalitis, and can suppress T cell activation (Tian et al., 2008).

1.6. Structure of Integrins

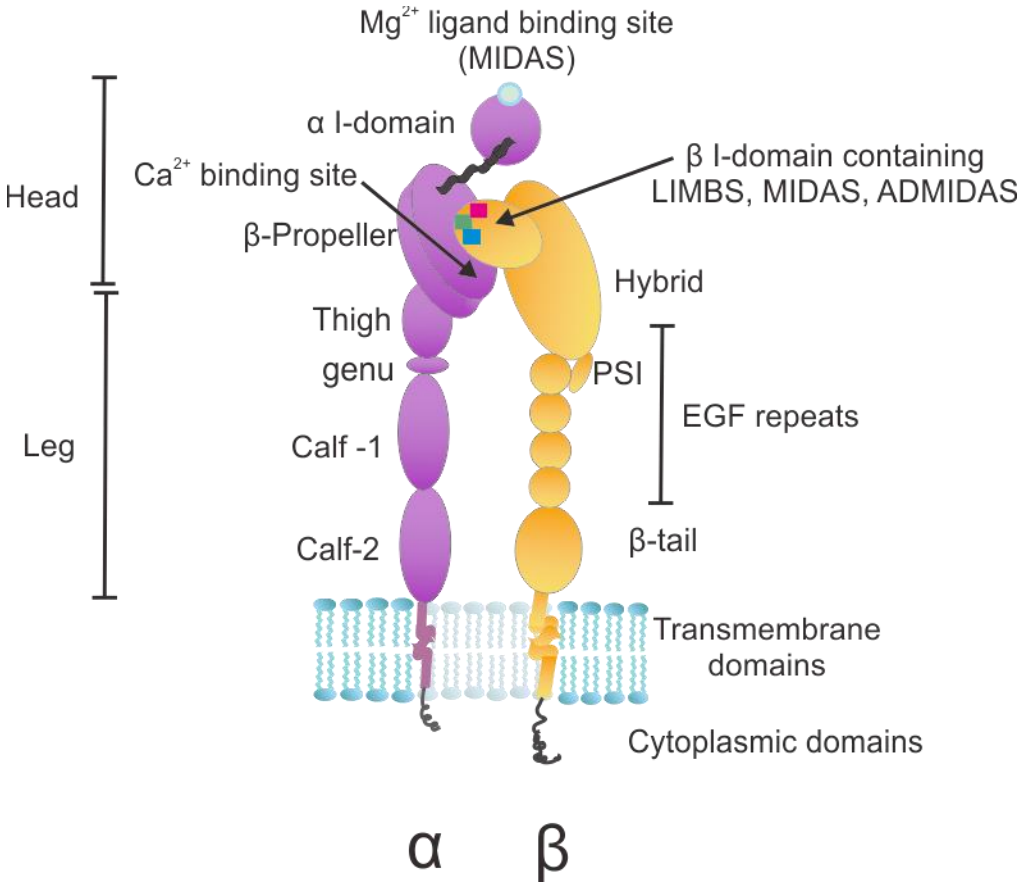


Figure 2. Structure of a fully activated integrin in detail.

The α-chain is shown in purple while the β-chain is shown in orange. The head of the α integrin consist of the β-propeller and α I-domain which has the main ligand binding site (MIDAS) while the head section of the β integrin consists of the β I-domain which contains the MIDAS site flanked by 2 calcium binding sites LIMBS and ADMIDAS, and the hybrid domain. The red square indicates the location of ADMIDAS, the green box indicates the location of MIDAS and the blue square represents the LIMBS (SyMBS) site on the β-chain.

The integrin α subunit has a seven bladed β-propeller unit supporting the integrin α head domain containing the αI-domain (Figure 2). An αI-domain is present in the α chain in all β2 family integrins. Calcium ions bind at EF-hands at the lower end of the β-

propeller facing away from the ligand binding site. For integrins to bind to ligands, Magnesium ions are required in the MIDAS (metal-ion dependant binding site) in the α chain (Valdramidou et al., 2008).

The integrin β chain consists of a PSI (plexin-semaphorin-integrin) domain, hybrid domain, four EGF (epidermal growth factor) repeats and a βI-domain. The β chain cytoplasmic domain connects the integrin to the cytoskeleton and multiple signalling pathways (Gahmberg et al., 2009, Barczyk et al., 2010). The βI-domain of the β chain also has a MIDAS (metal-ion dependant adhesion site) where magnesium ions bind for adhesion to occur and an adjacent site, ADMIDAS, where inhibitory calcium ions bind (Lee et al., 1995, Tan, 2012). Manganese ions bound to ADMIDAS lead to conformational changes associated with integrin activation. An additional calcium ion binding site is present at LIMBS (ligand induced metal binding site), also known as SyMBS (synergistic calcium binding site), which increases the binding affinity of magnesium ions at the MIDAS (Valdramidou et al., 2008, Campbell and Humphries 2011, Tan, 2012). The heterodimeric structure of integrin subunit pairs is formed between a surface of the β-propeller of the α chain and the hybrid domain of the β chain. In the inactive, closed conformation, the integrin is bent at the genu of the α-integrin and between EGF-1 and EGF2 of the β-integrin. (Evans et al., 2009) I-domains, which are the main ligand-binding sites in those integrins that contain them, are not found in all integrins. (Humphries et al., 2000, Barczyk et al., 2010).

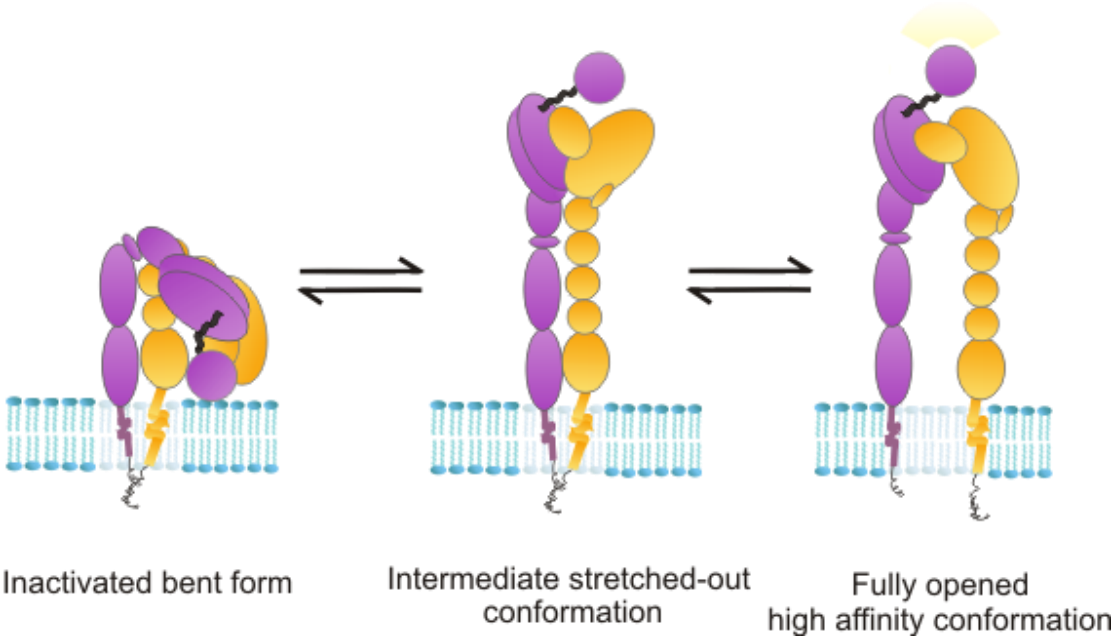


Figure 3. Dimeric structure of integrin during different activation stages.

On the left, integrin at rest has a bent, closed conformation while the right picture shows the open conformation. An integrin that receives a stimulus from chemokines or other surrounding stimulus stretches out and has intermediate affinity for ligands, as demonstrated in the middle. The structure on the right shows a fully activated integrin after stimulation, adopting an out-stretched open conformation where the 2 integrin chains are separated after the hybid domain swings open. This structure has high affinity for ligands. Other various forms of integrin structures could also be visualised during the transition from inactivated conformation to the intermediate conformation, and from the intermediate confirmation to the high affinity conformation (Xiao et al., 2004, Gahmberg et al., 2009). The α-chain is shown in purple while β-chain is shown in orange.

Integrins on unstimulated cells adopt a bent conformation when they are at rest and when the I-domain is unoccupied (Figure 3). Integrins bind to ligands at the α I-domain which activates the integrin (Luo at el., 2007). Using the blocking antibody 2D7 which bind to the N-terminus of the I-domain which includes most of MIDAS, it was determined that the low affinity integrin had an extended but closed structure. In the

same study, it was also shown using the M17/4 blocking antibody that is specific for the C terminus of the I-domain, that the high affinity integrin had an extended and open structure (Wang et al., 2009). Other inhibitory antibodies that can be used to study the integrin structure is CBR LFA-1/2 which locks the integrin in the stretched conformation while binding at the β I-domain and 7E4 which locks the integrin in the stretched but closed conformation by binding the hybrid domain, preventing the integrin from being highly activated (Ye et al., 2012). YTA-1 which binds to an epitope formed by both αL and β2 integrins can also be used for studies of LFA-1 binding (Zang et al., 2000, Byron et al., 2009). There are also antibodies that stimulate integrin activation such as MEM148, which binds to an exposed epitope which stabilizes the head piece and holds the integrin in its open conformation (Ye et al., 2012).

Integrin activity can be studied using monoclonal antibodies that bind to the activated integrin at specific sites and do not interfere with the ligand binding sites (Figure 4). However, these antibodies that are available to detected activated β2 integrins are only specific for human integrins. mAb KIM127 is used to detect integrins in the intermediate affinity, out-stretched conformation. This antibody binds to the I-EGF2 site on the β-integrin, which is hidden in the inactive, bent conformation. Another antibody used for studies of integrin activation is mAb 24, which binds to the β I-domain when conformational changes occurs as the hybrid swings out and the integrins adopts an open structure (Evans et al., 2009, Johansson et al., 2013).

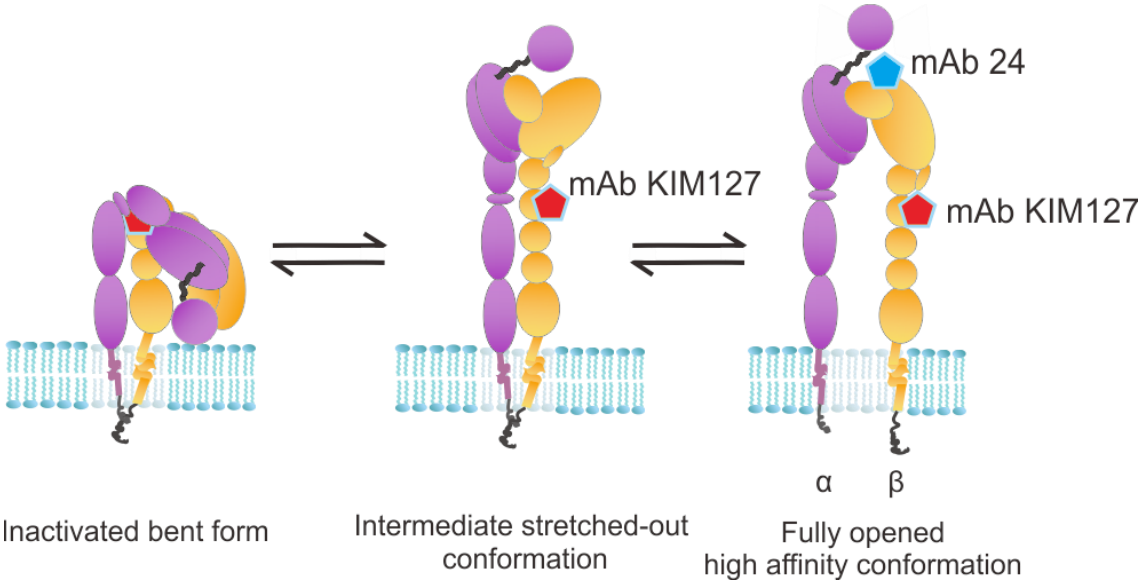


Figure 4. Antibodies used to detect human integrin activation states.

Adopted from figure 3, binding sites of antibodies that are used to detect different activation states of integrins are marked. mAb KIM127 that binds to I-EGF2 in the integrin intermediate affinity and high affinity state is marked with a red hexagon, mAB 24 which binds to β I-domain in high affinity state of the integrin is marked in blue.

There are also antibodies that can be used to detect active β1 integrins at different states of activation. N29 and 8E3 bind at the PSI domain when epitopes are exposed as the integrins are in the intermediate and high affinity state. HUTS-21 bind to the hybrid domain when it swings out to adopt the high affinity state and 9EG7 binds to the epitope on EGF-like domains that are exposed in the open structure of the high affinity state integrin (Byron et al., 2009, Johannson et al., 2013).

Integrin activation is dependent on cytoplasmic proteins such as talin and kindlin (Figure 5). Talin-1 exists a dimer and is the only isoform found in leukocytes and is the main cytoskeletal integrin activating protein in hematopoietic cells. Binding of the talin F3 domain to the β integrin membrane proximal cytoplasmic NPxY motif is required for

inducing LFA-1 extension to the intermediate affinity state. This requires increased levels of phosphatidylinositol 4, 5-bisphosphate (PIP_2) near the talin head (Alon and Feigelson, 2012). This binding event induces neutrophil slow rolling. To induce full activation of LFA-1 to the high affinity form, binding of both talin-1 at the membrane proximal cytoplasmic domain NPxY motif and kindlin-3 at the membrane distal cytoplasmic domain NxxY motif of the LFA-1 integrin are required to induce an open integrin headpiece and neutrophil arrest (Alon and Fegelson, 2012, Lefort et al. 2012, Moser et al., 2009).

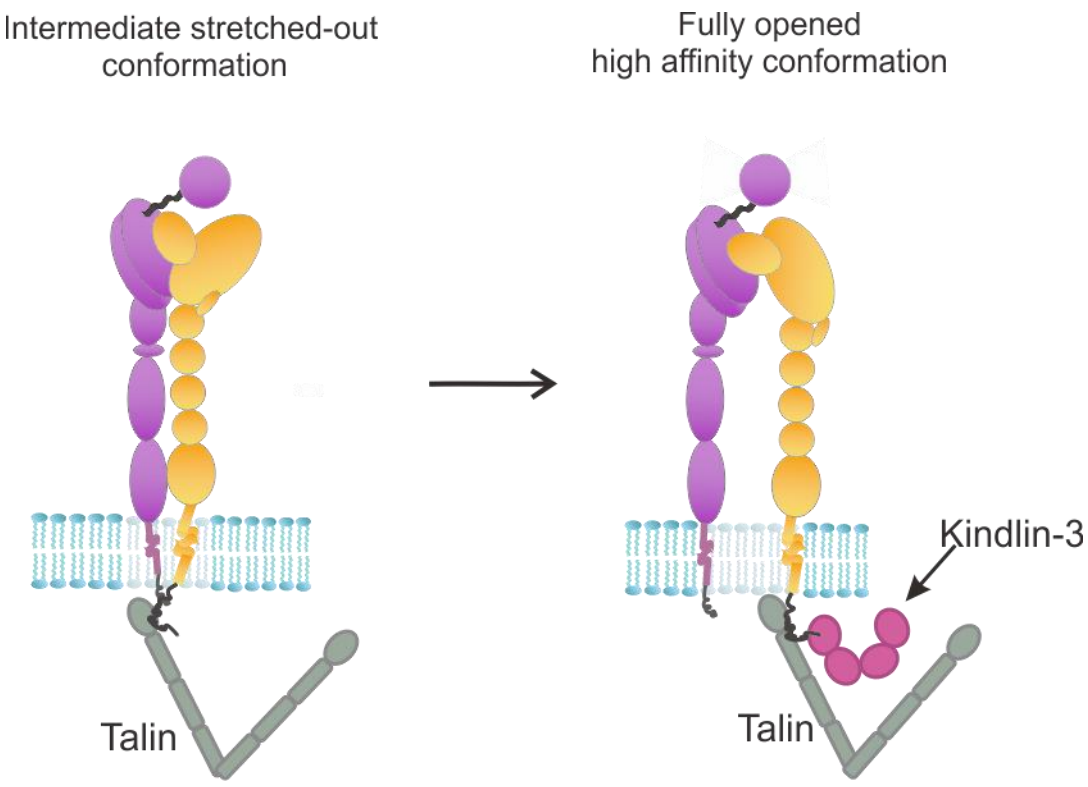


Figure 5. Interaction of talin and kindlin-3 with the integrin cytoplasmic domain at different stages of integrin activation.

1.7. Cell-cell adhesion: Affinity vs. Avidity

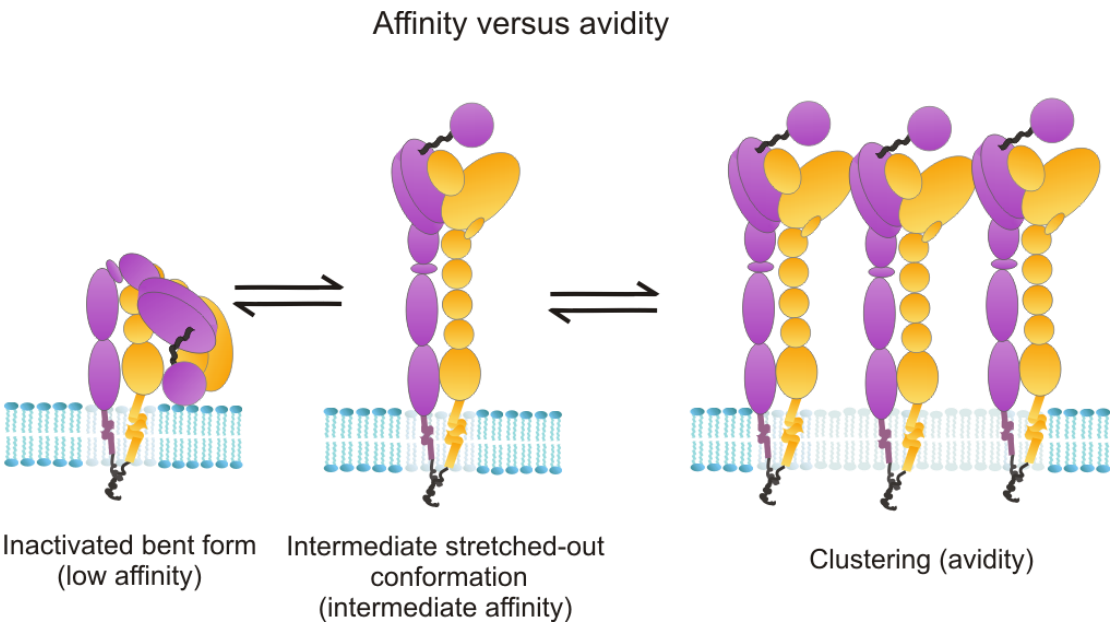


Figure 6. Modes of integrin activation for cell adhesion by increasing affinity or affinity.

Integrins in the resting, inactivated state have a closed and bent conformation. Upon receiving a stimulus from nearby receptors such as B/T cell receptors or G protein coupled receptors, integrins are activated and change conformation by extending to a stretched-out structure with intermediate affinity (Figure 6). A group of integrins can also cluster close together at membrane surface to increase total ligand binding abilities (Figure 6). The total strength of adhesion is attributed to both the strength (affinity) of individual integrin binding to the ligand and also by the density and arrangement of integrin-ligand binding (avidity or valency) (Carman and Springer, 2002).

1.8. Integrin functions in the Immune System

1.8.1. Formation of the Immunological synapse

Integrins are required for the formation of the immunological synapse between a lymphocyte and an antigen-presenting cell. After LFA-1 binds to ICAM-1 when a T cell engages with an antigen presenting cell, the TCR binds MHC/antigen in the periphery of the immunological synapse. More LFA-1/ICAM-1 interactions lead to the concentration of TCR/MHC interactions (which forms the central supramolecular activation complex, cSMAC) and better T cell signalling as Lck and Zap-70 are recruited (Grakoui et al., 1999). As the immunological synapse matures, LFA-1/ICAM-1 and talin can be found at the outer ring of the immunological synapse surrounding the T cell receptors (also known as the peripheral supramolecular activation complex, pSMAC). A ring of actin accumulates outside the pSMAC in the distal supramolecular activation complex (dSMAC). In the activation of cytototic $CD8^+$ T cell killing, cytolytic granules are released into the target cell within the pSMAC outside the cSMAC when the centrosome is polarised in the cSMAC (Dustin et al., 2010, Jenkins and Griffiths, 2010). The integrin/ICAM interactions in the immunological synapse are longlasting; integrin/ICAM-1 interactions could last up to 4 hours although the TCR/MHC interaction was not detected after 1 hour (Cambi et al., 2000).

Interestingly, memory T cells expressed more surface integrins than naïve T cells but integrins on naïve T cells can be activated rapidly to form transient interactions. The reason may be that memory T cells need to form strong and stable interactions via the immunological synapse in antigen presentation while naïve T cells need less integrins for efficient migration and scanning for antigens in lymph nodes (Pribila et al., 2004,

Jenkins and Griffiths, 2010). Due to the important role for integrins in T cell biology, T cell LFA-1/ICAM-1 interactions have been targeted in autoimmune disease and for the prevention of transplant rejection. For example, peptide inhibitors had been designed to block this interaction to prevent T cell activation (Anderson and Siahaan, 2003).

Integrins are also important for facilitating B cell activation. The activation of integrins enables B cells to undergo a rapid cell spreading and contraction response along the antigen-coated surface. Before the B cell is activated integrin and antigens are distributed uniformly across the cell surface. Inside-out signalling initiated by the B cell receptor leads to integrins clustering and formation of the B cell synapse. Integrin-adhesion to APC during the antigen recognition process allows for B cell activation when antigens are limited, by lowering the threshold of antigen required for activation of B cells (Carrasco et al., 2004, Arana et al., 2008).

1.8.2. Migration in response to a chemokine gradient

Interstitial leukocyte migration within lymphoid and peripheral tissue requires the ability of cells to adopt amoeboid features, the guidance by soluble chemoattractants during tissue injury or inflammation and the mobility along extracellular scaffolds (Friedl and Weigelin, 2008). Leukocytes that need to cross tissue barriers such as blood vessels and lymphatic vessels require integrins for 2D migration along the vessel walls. However, integrins are not strictly necessary for leukocyte migration once the cells are in 3D environments such as during leukocyte migration within tissues. Integrin ablated dendritic cells in peripheral tissues such as skin are highly motile and can use their actin cytoskeleton and myosin-II for the formation of protrusions and for contraction

necessary for tissue migration into sites of injury or infection and for entering lymphatic vessels (Lämmermann et al, 2008). Similarly, naïve T cell migration in tissues depend more on myosin-II than on integrin-ligand interactions (Jacobelli et al, 2010). However, this may change during inflammation, as effector cytotoxic T lymphocytes use αV integrins when they need to quickly migrate to sites of infection (Overstreet et al., 2013). T cells in lymph nodes can migrate up to 40μm/minutes within lymph nodes under the influence of chemokines in vivo (Smith et al., 2005).

1.8.3. Extravasation under shear flow

There has been an increase in studies investigating cell adhesion and migration under the influence of shear flow. How shear flow affects blood vessels has been looked at initially in the context of atherosclerosis, where blood vessels thicken due to plaque formation triggered by inflammation. Extravasation of leukocytes from the blood vessels into lymph nodes or tissues is mediated by selectin-ligand interactions to control the speed of cell rolling and integrin-ligand binding interactions that are able to resist the shear forces in the circulation (Figure 7, Palechek et al., 1997, Galkina et al., 2007, Kuwano et al., 2010).

Upon receiving signals from their cell surface receptors such as chemokine receptors, leukocytes in the circulation can modify the activity level of their integrins to a higher affinity form that can then bind ligands on the surfaces of blood vessels (Manevich-Mendelson et al., 2009). Integrins are rapidly activated by chemokines by a 3 step process in flow conditions, firstly by small contacts with the ligands on the endothelial surface as the leukocytes roll across the blood vessel walls, followed by integrin

extension by talin and opening of the integrin structure caused by ligand binding which is stabilised by low shear force and lastly, fully opened integrin structure with the separation of α and β subunits when talin and kindlin-3 binds to the cytoplasmic tail of integrins (Figure 5, Alon and Feigelson, 2012).

Shear stresses at arterial walls are maintained at approximately 8-15 dynes/cm^2 and can go up to 70 dynes/cm^2, while atherosclerosis occurs at 4 dynes/cm^2. However, the shear stresses in veins are much lower and have been described to range from 0.5 to 6 dynes/cm^2 (Papaioannou and Stefandis, 2005, Obi et al., 2008). Interestingly, shear flow modifies the expression of adhesive ligands and other molecules on endothelium. Laminar shear flow increased the expression of ICAM-1 on HUVECs (Human Umbilical Vein Endothelial Cells) and the adhesion of total leukocyte cells was also significantly enhanced. Although the expression of E-selectin was unaffected by shear flow, expression of Interleukin-1β was stimulated by shear flow which in turn upregulated expression of E-selectins on HUVECs. Interestingly, VCAM-1 expression was lowered in the presence of shear flow (Morigi et al., 1995).

Adhesion of neutrophils under shear flow is known to require β2-integrins (Lawrence et al., 1990, Jung et al., 1998). Interestingly, low shear stress has been suggested to stabilize integrin conformation during activation (Sigal et al. 2000, Astrof et al., 2006, Alon and Dustin 2007). Shamri et al 2009 showed that under shear flow, LFA-1 on rolling human peripheral blood lymphocytes (PBLs) adopted an extended conformation when interacting with chemokines bound onto the endothelium. The presented ICAM-1 ligands on the endothelium could then trigger instantaneous cell arrest under shear flow (Shamri et al., 2009). In neutrophils, PSGL-1 on the neutrophil binding to E selectin on the endothelium stimulates Src and Syk signalling that causes LFA-1 extension which is

necessary for cell rolling. CXCR2 on the neutrophil binding to CXCL1 on endothelial cells in turn activates Gαi which leads to LFA-1 adopting the open conformation, leading to cell arrest (Zarbock et al., 2007, Kuwano et al., 2010). Talin-1, exclusively found in leukocytes (Alon and Fegelzon, 2012), was found to be important for the extension of LFA-1 which is required for cell rolling while both talin-1 and Kindlin-3 were found to be necessary for neutrophil cell arrest in vivo (Lefort et al. 2012).

The leukocytes can then "crawl" along surfaces after activation (Figure 7). This process also allows lymphocytes to "flatten" themselves onto surfaces through rearrangements of the actin cytoskeleton, resulting in stronger adhesion, the ability to resist shear forces and allowing the cells to extravasate from the blood vessels and migrate through tissues to the sites of infections, under the chemoattractant influence of chemokines. These various processes such as actin remodelling, activation of integrins and selectins are tightly regulated (Jung et al., 1998, Nourshargh and Marelli-Berg, 2005, Gorina et al., 2013, Muller, 2013). Monocytes require both LFA-1 and Mac-1 for crawling. LFA-1 mediated crawling occurs in unstimulated venules but crawling becomes Mac-1 dependent during inflammation, which coincided with increased Mac-1 levels. Neutrophils only required Mac-1 for crawling in normal and TNF-α stimulated venules. Blocking of Mac-1 can reduce both crawling and extravasation of monocytes and neutrophils (Sumagin et al., 2010). Human peripheral blood T cells were also shown to require high affinity LFA-1 when crawling on endothelial layers while interacting with chemokines on the endothelium. Shear stress increased the formation of adhesive filopodia and some of these filopodia became invasive, enabling increased transendothelial migration (Shulman et al., 2009). The transition of cells from a state resisting shear stress in blood vessels to a state mediating migration in tissues to sites of infection is a fast process. The strength of cell adhesion may vary between these

different states, as migration in tissues may require lower adhesion than high-affinity adhesion under shear flow (Alon and Dustin, 2007).

Leukocyte transmigration can take between 2-5 minutes to get through endothelial cell layer and 5-15 minutes to get past the basement membrane, depending on the leukocyte cell type and the ratio of endothelial cells and pericytes in venular walls (Ley et al., 2007). Cultured endothelial cells display the formation of docking structiures or "transmigratory cups" when leukocytes are adhered, which are rich in ICAM-1 and VCAM-1 which initiate the transmigration process. These structures are also rich in ezrin, radixin and moesin and recruit cytoskeletal components such as vinculin, α-actinin and talin-l (Barrerio et al., 2002, Carman and Springer, 2004). The formation of transmigratory cups is also dependent on PIP_2 and RHO family of GTPases (Ley et al. 2007).

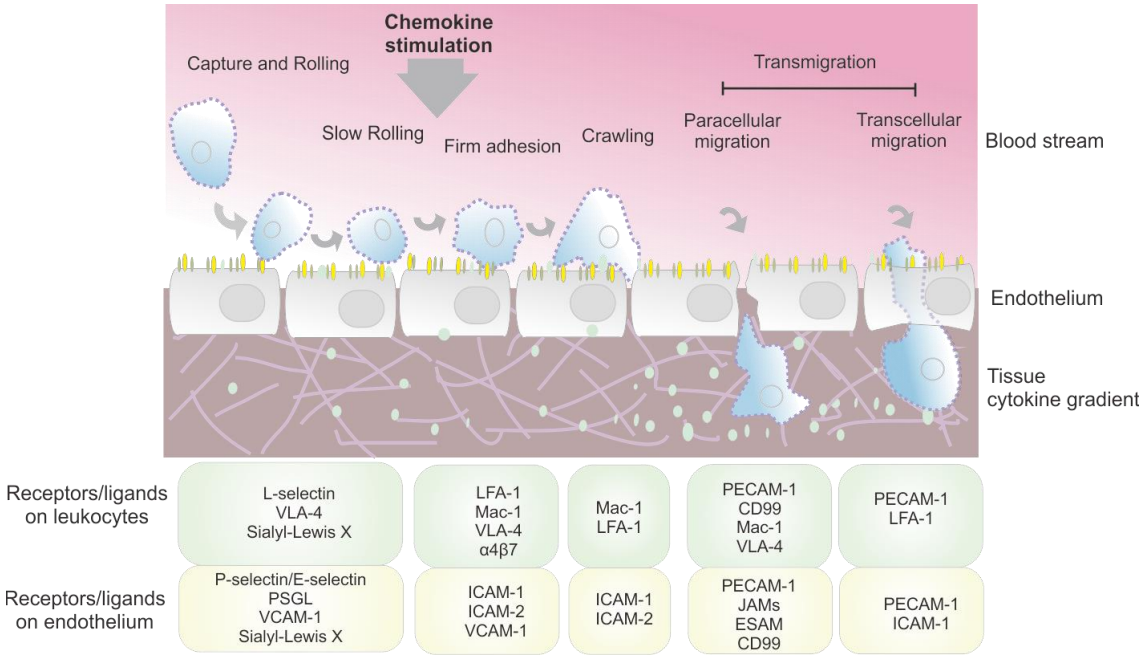


Figure 7. Paracellular and transcellular migration of leukocytes.

Diagram showing the process of leukocyte rolling, adhesion, crawling and transmigration on/through the endothelial cell surface. Two types of transmigration can occur: Paracellular migration (diapedesis) where leukocytes squeeze through tight junctions found between neighbouring endothelial cells, or transcellular migration where leukocytes are transported through an endothelial cell to the basal membrane and tissues. Receptors and ligands required for each stage in the exravasation process are marked.

Transmigration of leukocytes through the endothelial cell layer can occur either through a paracellular or transcellular route (Figure 7). The RHO family of GTPases in endothelial cells has been found to be important in the opening of junction contacts needed for paracellular transmigration. Myosin light chain kinase activity and subsequent endothelial cell contraction led to influx of calcium levels needed for the opening of cell junctions, and the process is regulated by ICAM-1 which in this context works as a signalling molecule (Rahman and Fazal, 2009). Molecules that do not support transmigration (such as VE-cadherin) will be directed away from the cell junction. Meanwhile, molecules that support transmigration are concentrated at the luminal surface. These molecules include PECAM-1 (Platelet/endothelial cell adhesion molecule-1) at the border of endothelial cells, JAM-A, JAM-B, JAM-C (Junction adhesion molecule), ICAM-1, ICAM-2, ESAM (Endothelial cell-selective adhesion molecule) and CD99 (Ley et al. 2007). These make up the endothelial lateral border recycling component (LBRC) from the membrane of interconnected reticulum of interconnected vesicles, which is also used during transcellular migration. The ICAM-1-rich LBRC surrounds the leukocyte and the recycling of LBRC is mediated by kinesin and microtubules (Mamdouh et al., 2008, Mamdouh et al., 2009).

Transcellular migration accounts for 5-20% of cell migration through cytokine-activated HUVECS (human umbilical vein endothelial cells). It is a fast process which takes less

than a minute to complete and it also uses LBRC (Cinamon et al., 2004, Ley et al., 2007, Mamdouh et al., 2009). In vivo, neutrophils utilise transcellular migration on thinning endothelial cell layers. PECAM-1 is also used for engulfment and transport of leukocytes during transcellular migration. Membrane protrusions first appear and ligation of ICAM-1 lead to the translocation of apical ICAM-1 to caveolae and F-actin rich regions. ICAM-1 was also translocated with caveolin-1 to the basement plasma membrane. These processes lead to the formation of a channel which is stabilised by actin and vimentin (Ley et al., 2007, Rahman and Fazal, 2009).

The preference of paracellular or transcellular migration is dependent on a variety of conditions, such as the source of endothelial cells, type of stimuli, type of leukocytes and tightness of cell junctions. Tight junctions in blood vessels and blood brain barrier leads to a preference of transcellular migration while leaky cell layers like postcapillary venules leads to increased paracellular migration of leukocytes (Rahman and Fazal, 2009). After crossing the endothelial cell layer, leukocytes need to go through the basement membrane and the pericyte sheath. The basement membrane is made up of laminin-8, laminin-10 and collagen type IV (Hallmann et al., 2005). Venular walls with patchy layer of pericytes also had low expression of matrix proteins such as laminin and collagen, which allowed easier migration of neutrophils and T cells with the formation of chemoattractant gradients. PECAM-1 induces β1 expression on neutrophils which binds to laminin while β2 engagement during neutrophil migration can stimulate β1 expression to allow migration in the extracellular marix (Ley et al., 2007).

In certain cases accessory cells appear to be necessary for leukocyte extravasation. B cells were found to cross the blood brain barrier via their interaction with monocytes on human brain endothelial cell layers. B cells were not able to transmigrate through the

endothelial cell layer on their own but were able to do so when monocytes were introduced. Therefore, it was suggested that monocytes stimulate B cells directly, thereby inducing cell adhesion to endothelial cells (Shurei et al., 2012).

Integrin-mediated migration is needed to overcome tissue barriers. For interstitial migration, cells such as dendritic cells can use integrin-independent modes of migration, where cells become amoeboid with frequent shape changes and migrate quickly by gliding. Weak integrin adhesion can facilitate migration by traction in narrow spaces (Pinner and Sahai, 2009). Adhesive structures are generally not observed during migration but large adhesion complexes are formed in the presence of inflammatory signals such as TNF-α to stop migration (Friedl and Wolf, 2010). 3D migration has been studied in chemotactic assays using a combination of fibrin and collagen I, which are ligands for β2- and β3-integrins, and β1-integrins, respectively, with soluble CCL19 to provide a chemokine gradient. Dendritic cells (both with and without integrin and talin) migrated via a blebbing action. This is driven by changes in the actin cytoskeleton in 3D (regulated by the Rho GTPase cdc42), where protrusions of actin flowing at the front of the cell is followed by squeezing actomyosin contractions at the trailing edge of the cell, driving the nucleus forward in narrow spaces (Lämmermann et al. 2009). B cells and granulocytes were also found to migrate in a similar manner. However, cells lacking talin or integrin were shown to be unable to migrate in 2D conditions, affecting the cells' ability to extravasate from the bloodstream to inflamed tissues (Lämmermann et al., 2008).

1.8.4. Phagocytosis

Phagocytosis has been defined as the process during which the cell recognises and engulfs particles larger than 0.5μm. It is an important mechanism in host defense where phagocytic cells such as macrophages clear pathogens and dead cells after they are opsonized. Phagocytosis is also important in tissue repair (Caron and Hall 1998).

Phagocytosis drives the reorganization of filamentous actin (F-actin) differently and is mediated by two types of receptors on macrophages: complement receptor 3 (CR3 or also αMβ2/Mac-1) and Fc gamma receptors (FcγRs). CR3 binds C3bi on complement-opsonized targets and C3bi-dependent uptake can occur without pro-inflammatory signals. Cross-linking of the integrin receptor CR3 activates only Rho and targets "sink" into the cell with little protrusive activity. In contrast, FcγRs bind to immunoglobulin G (IgG)–coated targets and uptake is accompanied by pseudopod extension and membrane ruffling. There is also activation of the respiratory burst by reactive oxygen species and production of cytokines, such as tumor necrosis factor–α. FcγRIIA cross-linking specifically results in the activation of Cdc42, which activates a Cdc42-Rac-Rho signalling cascade (Caron and Hall 1998, Dupuy and Caron, 2008).

Complement receptor 3 (CR3 or also αMβ2/Mac-1) has been identified as the major integrin involved in phagocytosis (Arnaout et al., 1983, Springer et al., 2005). During phagocytosis, talin was recruited to form phagosomes and activating Mac-1 integrin (Figure 8). The talin head has a FERM domain and α helical tail, which can exist in an "open" conformation state or "closed" autoinhibitory state. This "closed" autoinhibitory state of talin can be activated by increased PIP2 levels or by RIAM (Rap1 interacting adaptor protein). Talin activated by RIAM can then bind to the W747 and F754 sites of

the β2 integrin cytoplasmic tail of CR3 (αMβ2/Mac-1) in macrophages to activate the integrin (Anthis and Campbell, 2011). Macrophages and fibroblasts lacking talin or RIAM displayed impaired CR3-mediated phagocytosis but FcγR-mediated phagocytosis was not affected (Lim et al., 2007, Lafuente et al., 2013).

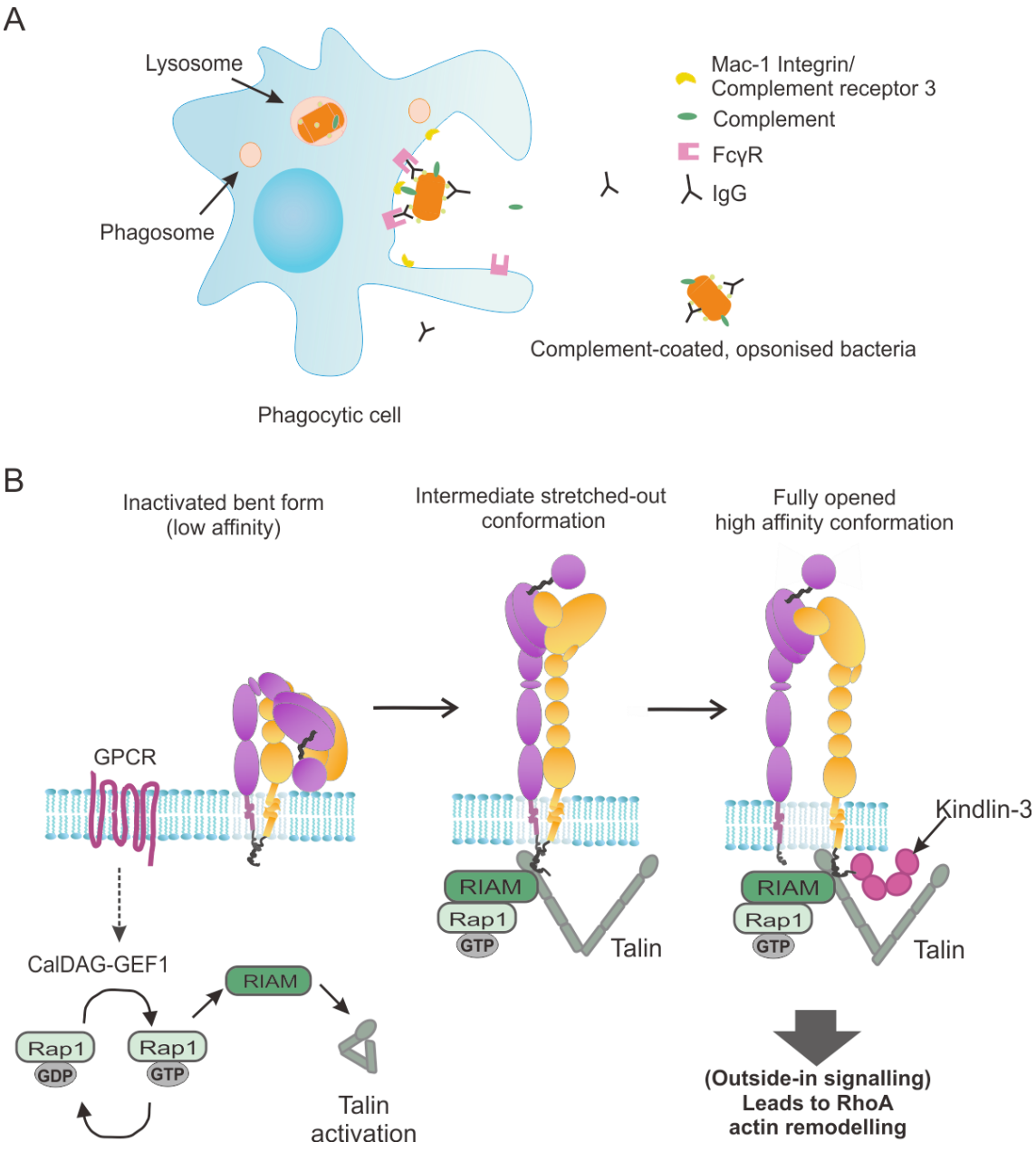


Figure 8. Interactions during the phagocytic process.

(A) A phagocytic cell such as macrophage or dendritic cell during integrin-mediated phagocytosis. Complement-coated, opsonised bacteria with IgG bound are taken up by the phagocytic cell, where IgG bound to the bacteria crosslink with the FcγR on phagocytic cell and Mac-1 integrin (complement receptor 3) binds to the complement C3bi deposited on the bacteria. (B) Chemokine stimulation through G protein coupled receptors leads to inside-out signalling to activate the Mac-1 integrin. Increased levels of Ca^{2+} *and diacylglycerol leads to acvtivation of CalDAG-GEF1 which activates Rap1. Active Rap1 that is GTP-bound can activate RIAM which interacts with talin. This allows talin to activate the integrin at the β-integrin cytoplasmic domain which results in the integrin adopting the intermediate affinity conformation. Kindlin-3 binding leads to the fully active integrin to bind to the ligand iC3b, which also allows outside in signalling to occur. Outside-in signalling involves actin remodelling via RhoA which is necessary in phagocytosis for the uptake of foreign particles.*

1.9. Bidirectional signalling of integrins

Integrins are bidirectional signalling receptors and are involved in "inside-out signalling" and "outside-in signalling" (Figure 9). Integrins can be activated by intracellular signals, such as those induced by chemokines or by neighbouring membrane receptors; this is termed "inside-out signalling" of integrins (Feng et al., 2012). Integrins undergo conformation changes as the extracellular domains stretches out to achieve the "intermediate-affinity" state. This can be achieved by directly targeting the cytoplasmic domains of integrins. The avidity from a cluster of integrins is also able to activate neighbouring integrins. As the extended integrins bind to the ligands, outside-in signalling can activate the open conformation of integrins leading to the "high-affinity" and active state. Outside-in signalling is defined as the steps after ligand binding. This further strengthens the integrin-ligand binding needed to facilitate

cell adhesion and formation, for example, of the immunological synapse between antigen presenting cell and T cell, cell migration, cytokine production and proliferation in T cells and degranuation of neutrophils. A common method to study outside-in signalling events is to use PMA or Mn2+ to bypass inside-out signalling events. (Zhu et al., 2007, Barczyk et al., 2010, Abram and Lowell, 2009).

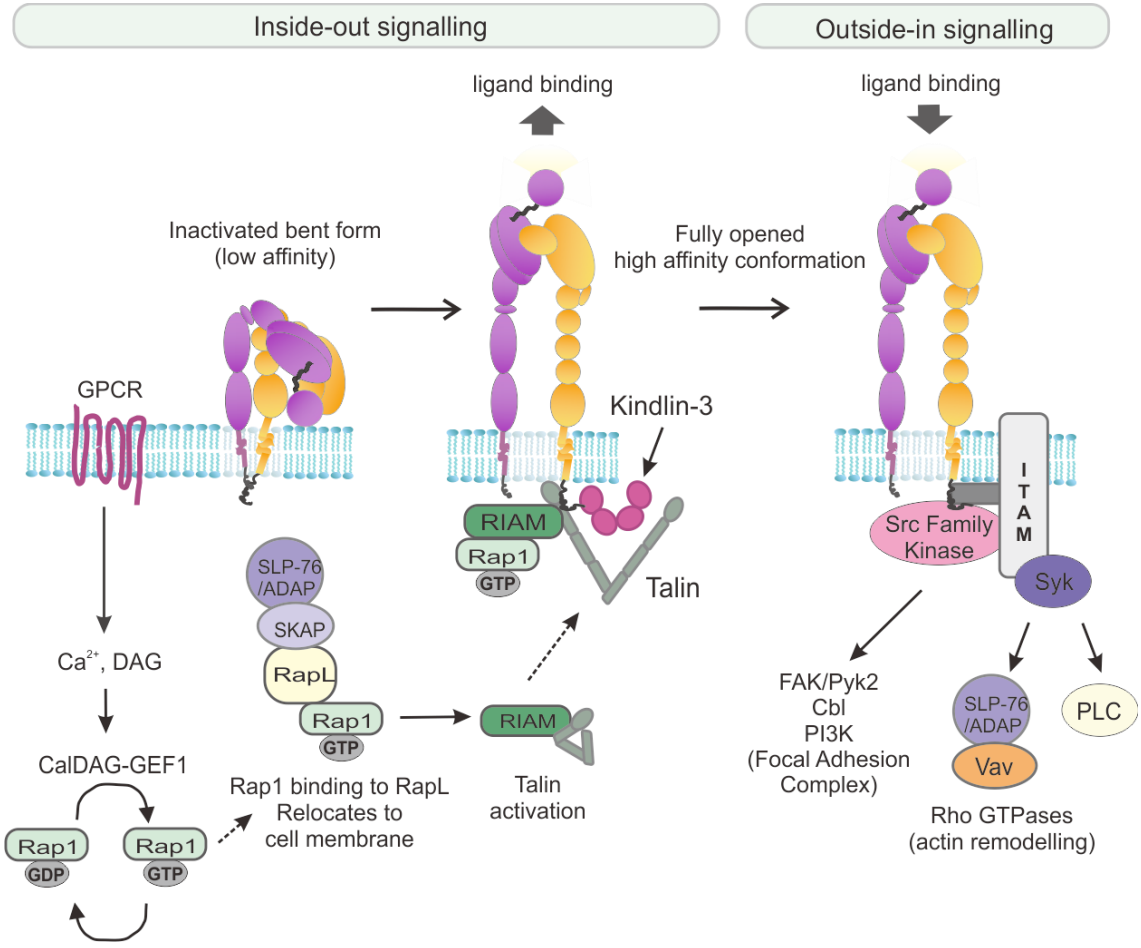


Figure 9. Inside out signalling and outside-in signalling.

Diagram depicting inside-out signalling and outside-in signalling. During inside-out signalling, G-protein coupled receptors are stimulated by chemokines that lead to the activation of GTP-bound Rap1. B/T cell recptors activation can also lead to production of Ca^{2+} and diacylglyerol production. Rap1 binds to RapL and recolates to the membrane where it binds to the SLP-

76/ADAP/SKAP complex, where SKAP1 has a binding site for RapL. Active Rap1 binds and activates Talin, which then binds cytoplasmic tail of β2-integrin tail leading to integrin activation. Kindlin3 leads to further activation of the integrins leading to firm adhesion. Outside-in signalling consists of events after ligand binding. Src family kinase such as Src is associated to the β2-integrin tail and when phosphorylated, becomes active and recruits Syk (in myeloid cells; Zap70 in lymphocytes) to ITAM (immunoreceptor tyrosine-based activation motifs) which is linked to the β2-integrin tail. Activation of Src lead to the formation of Focal Adhesion Complex by the activation of FAK/Pyk2, Cbl and PI3K. Syk is phosphorylated by Src which leads to activation of Phospholipase C (PLC) which is also a component of inside-out signalling. Syk phosphorylation also leads to actin remodelling regulated by Rho GTPase, activated by SLP70/ADAP and Vav (Evans et al., 2009, Jakus et al., 2007).

Changes in integrin conformation can increase affinity for ligands and integrins can also move closer together or cluster on the surface of cell membranes to increase avidity for the ligand binding. Clustering occurs during integrin diffusion, multivalent ligand binding or after inside out signalling. FMLP (Formyl-Methionyl-Leucyl-Phenylalanine), a bacteria-derived peptide and chemoattractant produced ar sites of inflammation, is able to induce LFA-1 clustering as well as integrin activation in neutrophils. It was also suggested that calpain hydrolyses talin to release integrins and are stabilised by α-actinin during clustering (Patcha et al., 2004, Saltel et al, 2009). Nanoclusters are necessary for signalling processes between cells (Whitlock et al., 2000) and promotion of focal adhesions (Humphries et al., 2007). Aside from increase in adhesion strength, clustering can facilitate outside-in signalling which can occur in phases. Early phases include integrins binding to ligands, causing lateral diffusion and recruitment of neighbouring integrins which involved talin, vinculin, paxillin and focal adhesion kinase. In the later phases, Src-dependent actin polymerization and myosin recruitment occurs, resulting in the formation of distant integrin clusters which moves inward to

form bigger adhesions via vinculin and myosin contraction (Humphries et al., 2007, Yu et al., 2011).

1.9.1. Inside-out signalling: Known pathways in integrin activation in lymphocytes

Integrins are activated by inside-out signalling. This activation can be triggered by G-protein coupled receptors after the leukocyte in the blood stream comes into contact with chemokines on the endothelium, with low shear forces stabilizing the extended integrin structure. Chemokines activate G-protein coupled receptors on leukocytes which can activate talin and Rac1/Rap1 which in turn regulate integrin activation. Once firm adhesion and signalling is established, actin remodelling occurs via Rho-GTPase activation (Figure 8B, Alon and Shulman, 2011, Alon and Feigelson, 2012). Chemokines activate Rho GTPase and PKCζ and these signalling components are important for LFA-1 integrin activation during in vivo homing (Giagulli et al, 2004).

Interestingly, in the presence of shear flow, also Rac1 was found to control chemokine-induced adhesion of T cells while Cdc42 negatively regulated this process (Bolomini-Vittori et al., 2009). Upstream of the Rho family small GTPases, the Janus kinase (JAK) family of protein tyrosine kinases (PTKs) have an important role in chemokine receptor signalling in T cells. The chemokine CXCL12 activates JAK 2/3 phosphorylation which in turn activates RhoA / Rac1 / PLD (Phosholipase D) / PIP5K1C (phosphatidylinositol-4-phosphate 5-kinase) upstream of Rap1 and the Rho-GEF, Vav-1, ultimately leading to the activation of LFA-1 and VLA-4 integrins (Montresor et al, 2013).

Crosslinking of the B or T cell receptor can also induce the activation of integrins which modulates the cell contact with the antigen presenting cell during immunological synapse formation. Lck is a tyrosine kinase which is activated by the TCR and is negatively regulated by CD45. Inhibition of Lck using PP2 inhibits LFA-1 binding to ICAM-1 in Jurkat T cells (Fagerholm et al, 2002). The Rap1 small GTPase is a critical integrin regulator and has been established to be important in the activation of integrins via inside-out signalling initiated by Diacylglycerol/Phorbol ester or through the T cell receptor and is dependent on Phospholipase C (PLC) (Katagiri et al., 2004). Rap1 can induce the formation of a Rap1/RIAM/ADAP/SKAP1 complex at the cytoplasmic tail of β integrins in T cells. This complex interacts with talin to activate integrins leading to stable T cell interactions with antigen presenting cells when T cells have been stimulated through the T cell receptor (Ménasché et al., 2007, Abram and Lowell, 2009). This Rap1/RIAM/ADAP/SKAP1 complex is also thought to be important in β2-integrin inside-out activation in T cells stimulated through the chemokine receptor CCR7 and also involves kindlin-3 or RapL (Katagiri et al., 2003, Kliche et al., 2012). Other signalling complexes that can form when the T cell receptor is stimulated is the Rap1/RapL/RIAM complex that interacts with talin, SKAP1 and ENV to activate LFA-1, or the SLP-76/ADAP/SKAP1 complex, which are further described below.

RIAM (Rap1-interacting adaptor molecule) interacts with Profilin and Ena/VASP proteins which are important regulators of actin dynamics (Lafuente et al., 2004). RIAM also binds to Rap1 and recruits talin to the complex, leading to integrin activation. ADAP (adhesion and degranulation–promoting adaptor protein), another adapter protein implicated in LFA-1 integrin regulation, is regulated by the Src kinase Fyn and together with SKAP1 are important in TCR-mediated signalling (Kliche et al., 2006). SKAP1 or Src kinase-asociated phosphoprotein 1 is also known as Skap55, and is thought to form

a SKAP1-RapL-Rap1 complex that binds to LFA-1 and mediates inside-out signalling during T cell migration (Raab et al., 2010). SKAP-HOM (SKAP-homologue) which is also a substrate of the Src kinase Fyn is also thought to be important in integrin activation and NK-κB signalling in T cells (Witte et al., 2012, Abram and Lowell, 2009). SLP-76 (76-kD src homology 2 domain-containing leukocyte phosphoprotein) is an adaptor protein which is also important for inside-out signalling to LFA-1 in T cells after TCR engagement (Horn et al, 2009). However, the exact details of how integrins are activated by inside-out signalling, such as the involvement of kinases and downstream interacting proteins after TCR engagement or chemokine signalling is yet to be clarified.

1.9.1.1. PLC

Phospholipase C (PLC) is involved in both inside-out activation of integrins and outside-in signalling leading to firm adhesion. Chemokines activate G-protein coupled receptors on leukocytes which can activate talin and Rac1/Rap1 which in turn regulate integrin activation. These receptors also regulate PLC and PI3K (Phosphoinositide-3-kinase). PLC in turn activates diacylglycerol (DAG) production and calcium signalling via inositol-3,4,5-triphosphate production. Calcium signalling has been shown to be important in integrin-mediated cell adhesion in neutrophils and calcium signalling leading to Rap1 activation is also necessary for inside-out activation of β2 integrins in T cells (Kanner et al., 1993, Hellberg et al., 1996, Alon and Shulman, 2011). T cells that were activated at the T cell receptor also activate Rap1 via PLC-γ1 (Phospholipase C gamma 1) (Katagiri et al., 2004).

Interestingly, in contrast to the situation in naïve T cells, effector T cell adhesion did not require chemokine stimulation for inside-out activation of integrins. A brief contact with high density of the LFA-1 and VLA-4 integrin ligands, ICAM-1 and VCAM-1 could activate integrins. PLC-γ1 is the major PLC isoform in T cells and was found to be hyper-phosphorylated at Tyr783 in effector cytotoxic T lymphocytes but not in peripheral blood T cells, and chemokine-independent effector T cell adhesion to integrin ligands was inhibited by PLC inhibitors (Shulman et al., 2012).

PLC is also important in outside-in signalling leading to integrin clustering by remodelling of the actin cytoskeleton. The Vav family of guanine nucleotide exchange factors is needed for the activation of PLCγ and calcium signalling after inside-out activation of integrins in B and T cells. Vav also affects PLCγ in neutrophils to establish firm cell adhesion under shear flow and oxidative bursts. It is thought that the SLP-76 adapter protein mediates the interaction between Vav and PLC (Abram and Lowell, 2009).

1.9.1.2. Involvement of AGC kinases in integrin signalling

AGC kinases consist of 60 serine/threonine protein kinases which are closely related to PKA (cAMP-depenent protein kinase 1), PKG (cGMP-dependent protein kinase) and PKC (Protein Kinase C) (Pearce et al., 2011). Some of these members include Akt (Protein Kinase B), S6K (ribosomal protein S6 kinase), PKC (Protein Kinase C), RSK (p90 ribosomal S6 kinase), and PKA (protein Kinase A). PDK1 (Phosphoinositide-dependent kinase-1) is the master regulator of this group of kinases, central to the regulation of AGC kinases. AGC kinase family members have been implicated in

integrin regulation in leukocytes. PKC is important for T cell signalling, and for regulation of the immunological synapse and cell polarity (Quann et al., 2011). PKC has also been shown to be necessary for cell signalling, adhesion and migration in neutrophils (Bertram et al., 2012).

Phosphoinositide 3-kinases (PI3Ks) produce inositol lipids that are implicated in the regulation of cell growth, proliferation, survival, differentiation and cytoskeletal changes (Vanhaesebroeck and Alessi, 2000). It was observed in B cells that the B cell receptor and IL-4 signalling activate Class 1A PI3K (p110δ) which lead to the phosphorylation of AGC kinases (Akt and downstream p70S6K, Erk) as well as FOXO3a, GSK3, calcium influx and PLCγ2 signalling (Bilancio et al., 2006). It is not known if these AGC kinases that were phosphorylated have any effect in integrin-mediated cell adhesion.

Phosphorylated Akt, ERK and JNK levels increased during CXCL13 and Sphingosine-1-phosphate induced chemotaxis. Integrin-mediated cell adhesion stimulated by CXCL13 and S1P was regulated by Rap1, while B cell migration and cytoskeletal arrangements were controlled by Rap1-dependent regulation of Pyk2 (Durand et al., 2006). PKCζ, downstream of PI3K, was also found to control avidity of LFA-1 on splenocytes in cell homing when triggered via chemokines (Giagulli et al., 2004).

Much of the research in the regulation of integrin activation has been carried out in T cells or cancer cell lines but not so much is known in B cells, especially primary B cells and how integrins in these cells are regulated during normal adaptive immunity. It is not clear if AGC kinases, or which AGC family members, are important in integrin regulation in B cells.

1.9.1.3. Phosphoinositide 3-kinase

Phosphoinositide 3-kinase (PI3K) isoforms are classified according to their substrate specificity and sequence homology. Class 1 PI3K have been extensively implicated in cell polarity and migration. PI3K is activated by G protein-coupled receptors and tyrosine kinase receptors to produce PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Vanhaesebroeck and Alessi, 2000). Resting cells have a basal level of PtdIns3. Subsequent phosphorylation will produce PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which are important for the activation of Akt (Vanhaesebroeck and Alessi, 2000). There are 2 subclasses of class 1 PI3K, both of which has a 110kDa catalytic domain and a small regulatory subunit. Class IA PI3Ks are activated by tyrosine kinases or Ras signalling initiated by B cell receptors or T cell receptors. The catalytic subunit can be either α, β, or δ and there are 5 regulatory subunits such as p85 (Cantrell et al., 2001). Class IB PI3K can be activated by G-protein coupled receptors and has a catalytic domain consisting of the p110γ isoform and either a p101 or a p84 regulatory subunit (Cain and Ridley 2012). In neutrophils, it has been shown that PSGL-1 interacts with E-selectin on the endothelial cell leading to activation of p85-p110δ PI3K through a Src/Nef-associated factor 1 (Naf-1) pathway in turn leading to β2 integrin activation (Wang et al., 2001).

The p110δ isoform of PI3K has been found to differentially regulate β1 and β2 integrin-mediated adhesion, spreading and diapedesis of monocytes to VCAM-1 or ICAM-1. Inhibition of p110δ PI3K leading to inhibition of both Rac1 and Cdc42 caused decreased β1-integrin-mediated adhesion to VCAM-1 but β2 integrin-mediated adhesion and migration on ICAM-1 was not affected as the β2 integrins were still conformationally active (Ferreira et al., 2010).

How PI3 kinase affects integrin-mediated adhesion is not well understood. Class 1 PI3K activate serine/threonine kinase PKB/Akt as the increased PIP3 production leads to changes in conformation of Akt. Akt is also known as PKB or RAC-protein kinase and is the most studied downstream target of PI3K. Akt kinases are found in the cytosol but when cells are stimulated, Akt moves to the membrane. Akt is then activated by PDK1 and PIP3 at the cell membrane, downstream of PI3 kinase. PIP3 also promotes PDK1 localisation to the cell surface, where phospholipids binding and activation loop phosphorylation at Thr308 of Akt occurs. In order to achieve maximum activation, phosphorylation within the carboxyl terminus at Ser473 of Akt is needed (Macintyre 2011).

PDK1 also phosphorylates PKCθ in T cells but this is not dependent on PIP3 signalling (Sonnenburg et al., 2001). PDK1 also activates RSK (p90 ribosomal S6 kinase) (Mora et al., 2004). Whether any of these pathways are important for lymphocyte adhesion is unknown at present.

1.9.1.4. AGC kinases - Protein kinase C and Protein kinase D

PKC enzymes are regulated by diacylglycerol and calcium which produced by PLC. Hydrolysis of PtdIns(3,4)P2 by phospholipase C produces inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate is released into the endoplasmic reticulum and binds to IP3 receptors which are also calcium channels. Calcium is then released from the ER, which together with diacylglycerol activates PKC. There are reportedly 13 subtypes of PKC enzymes and these can be classified into classical, novel or atypical PKCs depending on their activation requirements. Classical PKCs (cPKC),

consisting of α, β and γ subtypes, are calcium-dependent and require phosphotidylserine (PS) and diacylglycerol (DAG) for activation (Steinburg et al., 2008). Novel PKCs include δ, ε, η, θ, μ, subtypes, and are calcium-independent, but are regulated by phosphotidylserine and diacylglycerol. Lastly, the atypical PKCs, ζ, ι and λ subtypes do not depend on calcium or diacylglycerol for activation.

Specific PKC isoforms have been previously implicated in integrin regulation in lymphocytes (Katagiri et al, 2000, Letschka et al., 2008). In T cells, the major PKC isoform PKCθ and its effector RapGEF2 are critical factors in TCR signalling to Rap1, which affects LFA-1 binding to ICAM-1 (Letschka et al., 2008). PKCζ was found to be important in regulating SDF-1-mediated $CD34^+$ human progenitor cell (hematopoetic cell) adhesion, chemotaxis and cell polarization. PKCζ affected actin polymerisation which was necessary for cell adhesion under shear flow (Petit et al., 2005). In neutrophils, PKCζ has also been shown to be necessary for the regulation of the actin cytoskeleton and integrin-dependent cell adhesion (Laudanna et al., 1998). In fibroblasts, it has also been shown that PKCα and PKCε are directly responsible for actin reorganisation and spreading. Furthermore, these isoforms are involved in regulation of cell adhesion receptor trafficking (Kim and McCulloch, 2011). In addition, PKC has been implicated in both integrin inside-out and outside-in signalling in platelets (Abram and Lowell, 2007).

PKCβ has been identified as the major isoform of PKC present in B cells. PKCβ knock-out mice B cells have been found to fail to respond to BCR stimulation and have reduced T-cell-independent antibody responses (Su et al., 2002). PKCβ is indispensable for B-cell antigen receptor (BCR)-induced NF-κB activation and B-cell survival (Guo et

al., 2004). However, the role of PKC isoforms in primary B cell adhesion remains poorly understood.

In lymphocytes, PKC activation leads to the activation of Protein kinase D 2 (PKD2). PKD has previously been suggested to be important in regulating β1 integrin function via Rap1 in the Jurkat T cell line (Meideros et al., 2005). Whether PKC and/or PKD plays a role in integrin regulation in primary lymphocytes has not been determined.

1.9.1.5. Rap1

Ras proteins such as Rap1 are small GTPase proteins that are activated when bound to GTP and become inactivated when bound to GDP. This process is regulated by Guanine nucleotide exchange factors (GEFs) which mediate GDP exchange for GTP, and by GTPase-activating proteins (GAPs) which hydrolyse GTP to GDP. Rap1 was found to be an effective activator of LFA-1 (Katagiri et al., 2000). As previously mentioned, Rap1 interacts with RIAM (Rap1-GTP-interacting adaptor molecule) to promote integrin activation via talin (Lee et al., 2009). RapL was also found to associate with Rap1 as an effector molecule after Rap1 activation that led to LFA-1 binding to ICAM-1 at the leading edge of T lymphocytes (Katagiri et al., 2003). In T cells, PLCγ was found to be necessary for LFA-1 activation. Downstream of PLCγ, the major PKC isoform in T cells, PKCθ and its effector RapGEF2 are critical factors in TCR-induced signalling to Rap1, ultimately leading to LFA-1 binding to ICAM-1 (Katagiri et al., 2004, Letschka et al., 2008). Rap1A is found at the contact site between a T cell and an antigen-presenting cell and is thought to positively modulate the T cell response by

controlling strength of cell binding via β1 and β2 integrins (Katagiri et al., 2002, Sebzda et al., 2002).

Similar observations have been made in B cells as B cells lacking Rap1A displayed poor integrin-mediated cell adhesion (Duchniewitcz et al, 2006). Rac2 is thought to be important in regulation of Rap1 in B cells. B cells expressing active Rac2 upregulated adhesion. Additionally, B cells lacking Rac2 displayed defects in actin polymerization necessary for the formation of the immunological synapse after BCR engagement (Arana et al., 2008). Rap1B is the major isoform in B cells. Rap1B has been shown to be important for early B cell development, T-cell dependent humoral responses, migration towards chemokines and homing to lymph nodes, due to poor adhesion to ICAM-1 (Chu et al, 2008).

1.9.1.6. RIAM

RIAM (Rap1-GTP-interacting adaptor molecule) is a member of the MRL (Mig-10/RIAM/Lamellipodin) protein family. Its ability to act as a scaffold to bind to Rap1 and recruit talin to the plasma membrane where it binds to the cytoplasmic domain of β integrins is essential for integrin activation. It has been shown that RIAM is a downstream regulator of Rap1-dependent signalling as knockdown of RIAM affects Rap1-dependent cell adhesion. RIAM is also necessary for the recruitment of PLCγ to F-actin (Patsoukis et al., 2009). RIAM also plays a role in regulating actin dynamics by interacting with Profilin and Ena/VASP proteins. RIAM contains a RA-like (Ras association) domain, various proline-rich motifs and a PH (pleckstrin homology)

domain that can interact with inositol phospholipids during calcium release (Lafuente el al., 2004 and Lee et al., 2009, Patsoukis et al., 2009).

1.9.1.7. ADAP/Skap1

ADAP is an immune cell adaptor (also known as adhesion and degranulation-promoting adaptor protein or Fyn binding protein) which has a SLP-76 (76-kDa src homology 2 domain-containing leukocyte phosphoprotein) binding site and interacts with Skap1 in the activation of LFA-1 (Raab et al, 2010, Wang et al., 2009). ADAP is expressed primarily in many hematopoietic cells, but not in B cells (Wang and Rudd, 2008). ADAP/SLP-76 is important for F-actin polymerization and association of this complex during LFA-1 enhanced T cell polarization and IL-2 production. This LFA-1-ADAP polarization was dependent on src kinases, Rho GTPases, phospholipase C, and phosphoinositol 3-kinase. Cells lacking ADAP were found to have reduced adhesion, clustering and motility, with inability to recruit integrins at the immunology synapse and reduced T cell proliferation (Wang et al., 2009).

RapL in the cytosol binds to Skap1 during inside out signalling leading to translocation to the cell membrane, which then regulates colocalization of RapL/Rap1 to activate LFA-1. This regulates T cell adhesion, motility, and conjugation with dendritic cells in lymph nodes. SKAP-/- T cells showed poor adhesion and co-immunoprecipitation experiments using anti-Rap1 did not detect RapL in resting or SKAP-/- cells but this complex was detected in stimulated wild-type cells (Raab et al., 2010).

Skap1 is also known SKAP-55. While Skap55 is only expressed in T cells, SKAP-55R (related homologue protein) was found to be expressed in both B and T cells and regulates cell adhesion. In B cells, after phosphorylation of BCR ITAM (immunoreceptor tyrosine-based activation motifs), Syk phosphorylates SLP-76 homologue SL-65 in B cells which then binds to BTK (Bruton's tyrosine kinase), leading to phosphorylation of PLC2 and calcium signalling. This complex then interacts with SKAP-55R which leads to Rap1 association with VLA-4 and LFA-1 and cell adhesion (Wang and Rudd, 2008).

1.9.2. Outside-in integrin signalling: Known components in integrin outside-in signalling in immune cells

Integrin outside-in signalling is generally described as ligand-induced signalling leading to firm cell adhesion and changes in the actin cytoskeleton. It is often thought that outside-in signalling is an amplifier of signalling events in cells and is difficult to distinguish from inside-out signalling events. The Syk/Src family of tyrosine kinases are major components of integrin outside-in signalling and are activated after β1, β2 and β3 integrin activation. Syk is found in myeloid cells and platelets while ZAP70 is found in T cells and natural killer cells. Src family kinases consist of Src, Lyn, Blk, Fyn and Lck which are expressed in haematopoietic cells. Outside-in signalling of integrins is dependent on both cell type and site of inflamed tissue (Abram and Lowell, 2007, Hogg et al., 2011).

1.9.2.1. Src/Syc family kinases

The Src family of kinases are non-receptor tyrosine kinases that consists of Hck, Fgr, Lyn, Fyn, Yes, Src, Frk, Blk and Lck (Byeon et al., 2012). Stimulation of immune receptors such as the B cell receptor or T cell receptor and Fc receptors on mast cells and macrophages activate the Src family kinases, which phosphorylate ITAM (immunoreceptor tyrosine-based activation motifs) at the cytoplasmic tail of Fc receptors or B cell receptors, leading to Syk binding, activation of Syk and downstream effectors. Both Src family kinases and Syk kinase were found to be directly interacting with the cytoplasmic domain of β2 and β3 integrins (Abram and Lowell, 2009). Src was also suggested to facilitate clustering of integrins in high avidity integrin binding. High affinity integrin structure and ligand binding triggered Src-dependent outside in signalling to stabilize β2-integrin-mediated adhesion (Piccardoni et al., 2004).

Myeloid cells lacking Src kinases such as Hck, Fgr, and Lyn were found to be deficient in neutrophil degranulation and ROS production after β_2 and β_3 integrin activation binding (Mócsai et al., 2006, Baruzzi et al., 2008). Macrophages that are deficient in Src family kinases were unable to phosphprylate downstream effectors such as Vav and Cbl (Casitas B-lineage Lymphoma) and had defects in firm adhesion, cell spreading and cell polarization. Other downstream effectors of Src can include FAK (focal adhesion kinase) and the homolog Pyk2 in fibroblasts (Piccardoni et al., 2004, Baruzzi et al., 2008, Abram and Lowell, 2009). Src was also found to be involved in the integrin-mediated internalisation of Staphylococcus aureus in fibroblasts (Agerer et al., 2006).

There are two members in the Syk family of cytoplasmic tyrosine kinases, Syk (Spleen tyrosine kinase) and Zap70 ζ (zeta) chain–associated protein, which have a common

structure of two SH2 domains and a C-terminal kinase domain. Syk is expressed in both lymphoid and myeloid cells and plays a critical role in signalling from immune receptors. Syk has also been shown to signal from the C-type lectin Dectin-1 on dendritic cells, and from integrins on neutrophils, macrophages and osteoclasts (Fallah-Arani et al., 2008). Except for its role in integrin signalling, Syk is also important in B and T cell development. (Fallah-Arani et al., 2008, ng et al., 2010).

Zap-70 is also known as ζ (zeta) chain–associated protein of 70 kDa which is a kinase downstream of Src-family kinases in T cells. Zap-70, together with Lck, is constitutively associated with LFA-1. LFA-1 binding to ICAM-1 has been shown to lead to phosphorylation of Lck and Zap-70, which results in LFA-1 changing from the medium-affinity to the high-affinity state via inside-out signalling. In addition, phosphorylated Zap-70 is known to phosphorylate Vav-1 in a Vav-1-talin complex, releasing talin which then binds to the β1-integrin cytoplasmic tail of α4β1 (Evans et al., 2011). Zap-70 has also been shown to have an important role in firm adhesion of human T cells under shear flow and T cells lacking Zap-70 had slower cell migration on ICAM-1, although some of these experiments were done with relatively unspecific kinase inhibitors, piceatannol (Wang et al., 2010, Evans et al., 2011, Hogg et al., 2011). Zap-70 also has a role in T cell microtubule-organizing centre polarization towards the antigen-presenting cell and recruitment of signalling molecules such as PKCθ to the immunological synapse (Blanchard et al., 2002).

1.9.2.2. Cdc42

Cdc42 is one of the Rho GTPases responsible for actin remodelling. (Sid and Manser 2011) Phosphorylation of α4 chain of VLA-4 recruits 14-3-3 proteins which facilities the binding of paxillin to form a stable ternary complex. 14-3-3 in this complex aids in the localisation of activated cdc42 at the leading edge of lamellipodia (Deakin et al., 2009). During inflammation, migrating neutrophils require formation of the leading edge and a contracting uropod at the rear. Cells deficient in cdc42 suffered from defects in such cell polarization. Although cdc42 was activated at the front of the cell leading to actin polymerization, cdc42 is also involved in the redistribution of CD11b which modulates myosin light chain phosphorylation to suppress lateral protrusions at the neutrophil uropod (Szczur et al., 2009). Cdc42 is also capable of long-range signalling. WASp (Wiskott-Aldrich protein) activated by Cdc42 recruits microtubules at the uropod and clusters integrins, which mediates uropod stability (Kumar et al., 2012).

Phosphorylation of the cytoplasmic tail of the β2 integrin at Thr758 in LFA-1 activates the small GTPases Rac-1/cdc42 involved in actin rearrangement. These processes are important for cell adhesion and T cell co-stimulation and signalling. Transfection of LFA-1 expressing COS-1 cells with dominant negative cdc42 showed reduced cell adhesion and spreading on ICAM-1 (Fagerholm et al., 2006).

1.9.3. Integrin binding proteins at cytoplasmic tail (Cytoskeletal and adaptor proteins mediating integrin activation)

1.9.3.1. Talin

Talin connects integrins with actin and vinculin in the actin cytoskeleton (Critchley and Gingras, 2008). There are 2 types of Talin found in vertebrates. Talin-1 is the more studied isoform due to the availability of monoclonal antibodies to this protein. Talin-1 is a 270kDa actin-binding focal adhesion protein that exists as a homodimer, detected in peripheral focal adhesion in cells. Talin-1 can be activated by binding to phosphatidylinositol-4, 5-bisphosphate (PtdIns (4, 5) P_2) generated downstream of G protein coupled receptors and Rho-GTPase at the membrane surface (Alon and Shulman, 2011). Activation of talin is mediated by a PTB-like domain (phosphotyrosine-interaction) via a two-site interaction with the integrin β tails, which is normally functionally masked causing a reduction in binding affinity (Yan et al., 2001, Lee et al., 2009). The talin head domain binding to the cytoplasmic tail of β-integrins leads to a separation of integrin tails and allows the extracellular domain of integrins to adopt the activated structure (Calderwood et al., 1999, Kim et al., 2003, Fagerholm et al., 2005, Simonson et al., 2006, Wegener et al., 2007).

Talin binds to $\alpha L\beta_2$ which leads to the integrin adopting the intermediate affinity conformation and binding to ICAM-1 ligands in transfected cells (Li et al., 2007). Human T cells that are rapidly migrating on ICAM-1 have high LFA-1 levels at the uropod and low levels of LFA-1 at the lamellipodia. The middle section of the cell, also known as the focal zone contains high affinity integrins and talin is also concentrated in

this zone. In contrast, LFA-1 at the lamellipodia is in the intermediate affinity conformation (Smith et al., 2005, Evans et al., 2009).

In T cells, talin has been shown to be necessary for the stability of the immunological synapse and for cell arrest (Wernimont et al, 2009, Simonson et al, 2006). During cell activation, the naïve T cell and antigen-presenting cell require stable cell contacts in the form of immunological synapse. In this process, talin controls TCR-mediated LFA-1 clustering which is required for conjugation of the T cell with the antigen-presenting cell. Although the talin head domain is important in the activation of integrins talin heads were diffused throughout the T cell and weakly associated at the plasma membrane while talin rod domains were found to be concentrated at the contact zone between T cells and antigen presenting cells (Simonson et al., 2006). Interaction of talin-lacking T cells with antigen-presenting cells was transient but T cells were still able to signal. However, T cells lacking talin were found to have defects in contact-dependent arrest which resulted in poor homing to lymph nodes, and decreased T_H1 cytokine production and cell proliferation. LFA-1 polarization was unaffected by talin deficiency but F-actin polymerization and polarization of vinculin at the immunological synapse in the dSMAC was defective (Wernimont et al., 2011).

Talin-1 was also found to be expressed in B cells, platelets, dendritic cells and monocytes while Talin-2 could be found in human blood macrophages differentiated from monocytes. Talin-1 was also found to be concentrated in podosomes, which are adhesive structures found, for example, in macrophages (Praekelt et al., 2012). Talin-1 is crucial for VLA-4 and LFA-1 integrin-dependent mature B cell migration to lymph nodes in the presence of chemokine signals. Homing assays have shown that Talin-1 null B cells cannot enter lymph nodes nor return to bone marrow while a small

population of B cells lacking Talin-1 were still able to enter the spleen. The absence of Talin-1 leads to failure to activate inside-out signalling of LFA-1 and VLA-4 integrins by non-chemotatic canonical agonists and B cell receptor ligation. Although CXCL12 and CXCL13 can stimulate activation of VLA-4 in B cells lacking Talin-1 in physiological settings, Talin-1 is required for activating LFA-1 during extravasation (Manevich-Mendelson et al., 2009). Talin-1 is also required for differentiation of Marginal Zone B cells in the spleen as Talin-1-dependent VLA-4 and LFA-1 are needed for full entrapment and retention of these cells during the maturation process (Lu and Cyster, 2002).

Another important function of mature B cells in the immune system is the production of antibodies after encountering T-cell dependent antigen. When the exogenous T-cell dependent antigen KLH-TNP with complete Freund adjuvant was injected into the footpad of Talin-1 null mice, this led to a reduction in Talin-1 null B cells entering lymph nodes to mediate the primary and secondary immune responses when compared to WT mice. B cells were also not recruited to the spleen. Repeated administration of KLH-TNP led to a marked reduction of antigen-specific and hapten-specific IgG and IgM. Talin-1 therefore affects optimal humoral responses against T-dependent antigens (Manevich-Mendelson et al., 2009).

1.9.3.2. Kindlin-3

Kindlin-3 is a 75kDa hematopoietic cell-specific adaptor protein which is similar in structure to talin. Both proteins contain FERM domains (Yates et al., 2012). FERM refers to the protein module consisting of 4.1protein, erzin, radixin and moesin homology domains. FERM proteins are a superfamily of proteins that are about 300 amino acids in length. Many are cytoskeletal proteins that are involved in linking the actin cytoskeleton with the plasma membrane (Pearson et al., 2000). The difference between kindlin and talin is that kindlin has a pleckstrin homology (PH) domain inserted in FERM subdomain 2 (Yates et al., 2012). Kindlin-3 and talin act synergistically to activate integrins by binding to the cytoplasmic tail of the integrin β chain (Moser et al., 2009 and Barczyk et al., 2010). Kindlin-3 has also been shown to be important in Mac-1 outside-in signalling (Xue et al., 2013).

Kindlin-3 is a member of the kindlin family of focal adhesion proteins. Kindlin-2 is expressed in all cells and has an important role in myogenesis and angiogenesis. In contrast, kindlin-1 is only expressed in epithelial cells and keratinocytes and mutations in kindlin-1 leads to skin blistering in Kindler Syndrome patients (Ussar et al., 2006, Montanez 2008, Meves et al., 2009, Pluskota et al., 2011). Expression of kindlin-3 is mainly restricted to hematopoietic cells and some endothelial cells (Ussar et al., 2006, Bialkowska et al., 2011). Kindlin-3 is required for integrin-mediated leukocyte adhesion (Moser et al., 2009). Interestingly, mutations in kindlin-3 have been identified as the cause of the rare genetic disorder, leukocyte adhesion deficiency type III (LAD-III), where integrins are normally expressed but cannot mediate cell adhesion. Except for mutations causing loss of kindlin-3 protein expression**,** mutations in the kindlin-3 (FERMT3) gene which affect the pleckstrin homology domain and membrane

association of the protein have been described in LAD-III patients (Svennson et al., 2000**,** Malinin et al., 2009, Manevich-Mendelson et al., 2009).

Mutations in kindlin-3 affect T and B cell adhesion and migration in Leukocyte Adhesion Deficiency (LAD-III) patients (Svensson et al., 2000**,** Manevich-Mendelson et al., 2009, McDowall et al., 2010, Hart et al., 2013). Using Kindlin-$3^{+/+}$, Kindlin-$3^{+/-}$ and Kindlin-$3^{-/-}$ chimeric mice, Moser et al has shown that kindlin-$3^{-/-}$ leukocytes displayed poor integrin-mediated adhesion to TNFα-stimulated cremaster muscle venules (Moser et al., 2009). It was also demonstrated that kindlin-3, together with talin-1, was important for the full activation of LFA-1 to the stretched-out and open conformation and neutrophil arrest under shear flow conditions. In contrast, talin-1 was enough for LFA-1 extension resulting in the intermediate affinity conformation and allowing neutrophil rolling. Therefore, it was suggested that talin-1 and kindlin-3 serve distinct functions in LFA-1 activation (Lefort et al. 2012). In contrast, Kindlin-3 was not necessary for VLA-4 activation by chemokines (Manevich-Mendelson et al., 2009).

It was shown that the PH-domain in kindlin-3 was necessary for B and T cell adhesion. Kindlin-3 was shown to be recruited to the B cell membrane when activated. An intact PH-domain in Kindlin-3 was necessary for PI(3,4,5)P3 to bind to the protein, possibly to strengthen outside-in signalling of LFA-1, leading to B cell adhesion and migration on ICAM-1 (Hart et al., 2013). It was also recently shown that kindlin-3 binding to the β2-integrin required the triple threonine phosphorylation site in the β2 integrin cytoplasmic domain (Morrison et al, 2013). This interaction was required in $CD4^+$ T cells for firm integrin-mediated adhesion in static and shear flow conditions and for T cell homing in vivo (Morrison et al., 2013). T lymphocytes lacking kindlin-3 were unable to spread on ICAM-1 surfaces or dendritic cells using LFA-1 when stimulated at

the TCR (Feigelson et al., 2011). However, the kindlin-3/integrin interaction does not appear to be crucial for T cell activation in vivo (Morrison et al, 2013). It was also demonstrated that kindlin-3 was not needed for effector T cell diapedesis even though it was necessary for cell adhesion and interstitial motility (Cohen et al., 2013).

1.9.3.3. 14-3-3

Phosphorylation of integrin cytoplasmic tails is important for the binding of integrin regulators and the regulation of cell signalling events. When T cells are stimulated by ligation of the T cell receptor, the β2 integrin chain of LFA-1 is phosphorylated at Thr758 in the cytoplasmic domain. 14-3-3 proteins are one type of regulator that has been identified to bind to phosphorylated integrins (Takala et al., 2008). 14-3-3 proteins are a family of approximately 30kDa ubiquitous small adaptor proteins present in all eukaryotic cells. 14-3-3 proteins are highly conserved and more than 200 binding targets have been identified that bind to 14-3-3 proteins. 14-3-3 proteins exist as dimers and are bound strongly to phosphorylated Serine or Threonine sequences in proteins at specific binding motifs, (S/x)pS/TxP or RxxxpS/TxP). Although these binding motifs are optimal for strong binding, there are binding sites found in many proteins that do not meet these requirements (Fagerholm et al., 2004, Legate and Fassler, 2009).

After the binding of 14-3-3 binding to the phosphorylated site of β2-integrin, Rac1/Cdc42 are thought to be activated to induce actin cytoskeleton reorganization to strengthen the contact between T cells and antigen presenting cell via the guanine nucleotide exchange factor Tiam1. Activation by SDF-1 also had the same effect in activating LFA-1, which suggested that this process may mediate activation of integrins on lymphocytes during cell adhesion and extravasation (Nurmi et al., 2007, Grönholm

et al., 2011). Blocking of 14-3-3 and mutation of Thr758 site which prevents the binding of 14-3-3 lead to reduction of COS cell adhesion to ICAM-1 (Fagerholm et al., 2005).

1.9.4. Remodelling of actin cytoskeleton

Integrins are important for connecting extracellular components with the actin cytoskeleton. These interactions can result in active remodelling of the cytoskeleton necessary for cellular functions such as adhesion and phagocytosis (Dupuy and Caron, 2008). Actin dynamics involve the assembly of G-actin at one end and disassembly of F-actin at the other end. Actin can be assembled in short branched filaments or unbranched, long straight filaments depending on interactions with Arp2/3 or formin (Pruyne et al., 2002, Goode and Eck, 2007).

The Rho family of proteins is important for actin remodelling. The Rho family of proteins consists of RhoA, RhoB, RhoC, Rac1, Rac2 and Cdc42. These proteins are inactive when GDP-bound and active when GTP-bound (Sid and Manser 2011). Rho is involved in stress fibre formation, formation of focal adhesion, cell morphology changes, cell aggregation, motility, membrane ruffling, smooth muscle contraction, neurite formation and cytokinesis. Rac is involved in membrane ruffling, cell motility, actin polymerization and cadherin-mediated adhesion. Cdc42 is involved in filopodia formation and cell-to-cell adhesion (Kaibuchi et al., 1999). Some downstream targets of Rho include Rho-kinase/ROK/ROCK and myosin light chain kinase/phosphatase (MLCK). The coiled coil ROCK (Rho Kinase) activates actin and myosin contraction to form stress fibres (Vardouli et al., 2005).

Downstream targets of Rac/Cdc42 include p21-activated kinase (PAK) and WASp/N-WASp (Wiskott–Aldrich syndrome protein). Activation of Wiskott–Aldrich syndrome protein WASp/SCAR, in turn activates the WASp-family verprolin-homologous protein (WAVE) proteins. WASp proteins contain VCA domains which are made up of a C-terminal Verprolin homology domain, a Cofilin homology domain and an Acidic domain. The VCA domain binds an actin monomer to an Arp2/3 complex, leading to a burst of nucleating actin polymerization (Higgs and Pollard, 2000, Takenawa and Suetsugu, 2007). Interestingly, although both Rac1 and Cdc42 are important in the formation of actin-rich structures, Rac1 was found to be regulate adhesion of T cells while Cdc42 negatively regulates cell adhesion. (Fagerholm et al., 2006, Bolomini-Vittori et al., 2009).

Some other important proteins which regulate actin dynamics include, for example, p58 β-PIX, which is a guanine nucleotide exchange factor that can regulate the actin cytoskeleton and cause membrane ruffling (Lee et al., 2001). Cofilin is an actin-binding protein which when bound to actin will cause depolymerisation, while profilin enhances nucleation of actin polymerization by activation of Cdc42, which in turn activates WASP and the Arp2/3 complex (Yang et al., 2000, Vardouli et al., 2005, Sit and Manser, 2011). Moesin acts as a crosslinker between the plasma membrane and actin and interacts with ICAM-3 at the uropods in T lymphocytes (Serrador et al., 1997).

Actin rich structures that are formed due to active rearrangements of the actin cytoskeleton in lymphocytes contribute greatly to their adherence and migratory functions. Membrane ruffling is associated with dynamic formation of lamellipodia and filopodia via active actin polymerization and depolymerisation in response to

extracellular signals (Kaibuchi et al., 1999). Lamellipodia, which are actin projections that help cells move and migrate, are mainly regulated by Rac1 proteins which are activated by WASP. The finer projections called filopodia are regulated by Cdc42, which is activated by WAVE/SCAR. Rho proteins regulate the formation of stress fibres and focal adhesion complexes by formins. Formins (or formin homology proteins) initiates filament assembly and appear to be activated by Rho GTPases and additional factors. While formins persistently associate with the fast-growing barbed end to enable rapid addition of actin subunits, these proteins also protect the ends from capping proteins (Goode and Eck, 2007).

Focal Adhesion Kinase (FAK) is a regulator of cell migration which connects extracellular matrix and the cytoskeleton via integrins. When FAK is phosphorylated at Tyr397, Src Kinase binds to FAK at Tyr397 which phosphorylate α-actinin at Tyr12 to form a complex that controls cell migration. Phosphorylated α-actinin has low affinity for actin crosslinking to form actin bundles, while dephosphorylation of α-actinin by PTP 1B (Phosphatase 1B) increases formation of focal adhesion plaques and clustering of integrins. However, phosphorylated α-actinin binds with Src, reducing Src binding to FAK, demonstrating that PTP 1B and α-actinin provided a feedback loop in regulating cell migration (Kaibuchi et al., 1999, Corgan et al, 2004, Zhang et al., 2006). Myosin contractility also plays an important role in cell migration. Myosin acts as a crosslinker which is regulated by phosphorylation of the light and heavy chains (Vicente-Manzanares et al, 2009.). Neutrophils utilize myosin contraction at the uropods for cell migration (Eddy et al., 2000).

1.9.4.1. α-actinin

The actin in the cytoskeleton can be arranged to form a mesh network or long F-actin bundles. Integrin signalling can stimulate the formation of the mesh network of actin through talin, vinculin and α-actinin. α-actinin belongs to the spectrin family of cytoskeletal proteins. 6 isoforms of α-actinin has been described and are grouped according to if they are muscle isoforms or non-muscle cytoskeletal isoforms. The non-muscle cytoskeletal isoforms are calcium sensitive. Isoform 4 is concentrated in circular dorsal ruffles and focal contacts whereas isoform 1 is found on stress fibres. α-actinin forms homodimers with a role in actin cross-linking and can be found along actin fibres and at adhesion sites. The interaction with the cytoskeleton is important in cell adhesion and migration (Sjöblom et al., 2008).

New data suggests that α-actinin is important in mechano-sensing at focal adhesion complexes in cultured fibroblasts (Columbelli et al., 2009, Shams et al., 2012, Roca-Cusachs et al., 2013). In early adhesion complexes, talin is bound to and activates integrins, and connects β3 integrins to the actin cytoskeleton. α-actinin has been found to compete with talin for binding to integrins. External force is transmitted via α-actinin to strengthen (mature) adhesions, and increased external force leads to recruitment of more α-actinin to the adhesion sites. This allows the cells to react to the force that they are subjected to. In mature adhesion complexes, β1 integrin-mediated cell adhesion takes over and α-actinin was observed to cooperate with talin (Roca-Cusachs et al., 2013).

1.9.4.2. Filamin

Filamin is a 280-kDa rod-shaped protein that crosslinks actin, forming either loose microfilament networks, or tight actin bundles and coordinates the interaction between integrins and the cytoskeleton. Filamin binds to the Thr758 site of the β2-integrin when it is unphosphorylated (Takala et al., 2008). It requires binding to the NxxY motif on β-integrins which coincides to where talin also binds to during integrin activation. Switching between talin and filamin determines integrin activation (Kiema et al., 2006, Boucard et al., 2013). In addition, knockdown of filamin in T cells increases cell adhesion to ICAM-1. Therefore, filamin acts as a negative regulator of talin-dependent integrin activation in T cells (Takala et al., 2008).

Filamin A is expressed in all cell types and is the most abundant isoform, found concentrated below the cell membrane and at adhesion sites during cell spreading (Kim et al., 2008). Filamin A can bind to both PKCα and PKCε isoforms of PKC and these interactions are important in early events of cell adhesion (Ng wt al., 1999, Ivaska et al., 2002). The formation of lamellipodia for migration of fibroblasts was also dependent on filamin and PKCζ (Nomachi et al., 2003). Filamin also affects immune cell function as filamin deficient T cells are unable to translocate PKCθ to the interface between T cell and antigen presenting cell, leading to deficient expression of Interleukin-2 by activated T cells (Hayashi and Altman, 2006, Kim and McCulloch, 2011). It has been shown that β7 integrin tails which bound more strongly to filamin mediated much worse cell migration and focal adhesion formation than β1 integrin tails, which had a preference for talin binding. Filamin binding was suggested to impair the membrane protrusion and cell polarization needed for cell migration (Calderwood et al., 2001, Bouvard et al., 2013).

1.10. Degradation and recycling of integrins

After ligand binding, integrins on cells can be deactivated or shed quickly like other adhesion molecules such as selectins and members of the immunoglobulin families. It has been found that L-selectin shedding was necessary for leukocyte migration after leukocyte/endothelial interaction during the extravasation process (Hafezi-Moghadam et al., 2001, Lee et al., 2007). Leukocytes shed LFA-1 after transendothelial migration and it is thought that this process is necessary in the regulation of outside-in signalling (Evans et al., 2006, Zen et al., 2011). Shed LFA-1 detected in blister fluid and synovial fluid contained the heterodimer intact headpiece with I and I-like domains but the CD11a central region epitope, G25.2 was absent. The CD11a chain was truncated to 110kDa while CD18 was minimally truncated to 80kDa and still in the highly active ligand binding conformation which can bind to ICAM-1. “Stubs” were found on neutrophils and was thought to be where LFA-1 was cleaved (Evans et al., 2006). Mac-1 integrins were also shed by macrophages during exit from sites of inflammation. These integrins that were shed were also found to be in the active ligand binding state and can bind to ligands such as ICAM-1, fibrin and collagen and were thought to act as soluble agonists (Gomez et al., 2012). CD18 was also found to be proteolytically cleaved from neutrophils in the presence of shear stress. This may promote pseudopod retraction and keep the neutrophil spherical shape leading to an increase in velocity and a reduction in microvascular resistance caused by erythrocytes (Fukuda et al., 2003).

Another important mechanism contributing to cell migration, together with integrin activation and deactivation is the endocytic trafficking of integrins. The availability of membrane receptors such as integrins is thought to be complementary to integrin affinity in the regulation of cell migration (Steinberg et al. 2012, Jacquemet et al.,

2013). Endocytosis and vesicular trafficking is thought to be important in controlling cell migration indirectly by regulating focal adhesion complexes or directly via regulation of integrins (Carman and Springer 2003). On the other hand, integrin trafficking was also thought to indirectly affect Rho GTPase signalling during cytokinesis during cell polarization and affect cell migration. Integrins can also drive trafficking of other receptors to facilitate cancer invasion and angiogenesis (Casswell et al., 2009).

Endocytosis of integrins occurs by clathrin-dependent and clathrin-independent endocytic mechanisms (Caswell et al., 2009). After endocytosis, integrins are transported to early endosomes which are vesicles where sorting by nexin proteins occurs, to determine whether receptors are sent to late endosomes and lysosomes to be degraded or are recycled back to the surface (Worby and Dixon, 2002). Most integrins are not degraded but are recycled to the plasma membrane via RAB4 (Ras-related proteins in brain GTPases)-dependent route or transported to the perinuclear recycling compartment before returning to the plasma membrane via RAB11 and/or ARF6 (ADP-ribosylation factor 6) interactions. However, not much was known at that time about β2 integrin recycling or how these heterodimers are degraded (Casswell et al., 2009).

Proteins in eukaryotic cells can be degraded by lysosome proteases, calpain, ATP-ubiquitin-proteasome-dependent processes or ATP-independent non-lysosomal processes. The major proteolytic process was thought to be ATP-ubiquitin-proteasome-dependent process as 20S proteasome, which is the core of the 26S complex, was most abundant in the cytosol and nucleus. Proteins that were internalised were tagged with ubiquitin, which acted as a degradation signal for this process. TPA (phorbol myristate acetate) induced poly-ubiquitination and rapid degradation of LFA-1 integrins in HL-60

cells. However, cells pretreated with ubiquitin-proteasome inhibitors had accumulation of poly-ubiquitinated proteins in the membrane fraction and the avidity of LFA-1 integrins was suppressed, suggesting that integrins can be degraded by this major proteolytic process (Katagiri et al., 1999). Epiblasts required talin for β1 integrin stability and focal adhesion stability. In recent research, the use of proteasome inhibitor MG-132 prevented β1 integrin degradation in talin null epiblasts. However, ubiquitination of β1, α5, or α6 integrins in epiblasts were undetectable, suggesting that α5β1 and α6β1 integrins may not be directly degraded by ubiquitin-proteasome pathway in embryoid bodies. Chloroquine (a lysosome inhibitor) also managed to rescue some integrin levels, suggesting that integrins could also be degraded by the lysosomal pathway (Liu et al., 2011).

1.11. Sorting Nexin Family

Sorting nexins are a family of at least 27 members of trafficking proteins containing a phospholipid-binding PX-domain. These molecules are hydrophilic and reside in the cytoplasm. Sorting Nexins are responsible for directing membrane proteins (which are bound to the PX-domain or associated through other protein-protein interactions) to lysosomes, the trans-Golgi network or back to the plasma membrane (Worby and Dixon, 2002, Knauf at al., 2005). Sorting nexin-4 (SNX4) is found in the periphery of endosomes and in the endoplasmic reticulum compartment near the nucleus. It was demonstrated in HeLa cells that sorting nexin-4 interacts with the transferrin receptor (TfnR) in early endosomes to divert the receptor away from lysosomal degradation (Traer et al., 2007). Another nexin that has interaction with adhesion molecules is nexin-17, which interacts with P-selectin. P-selectins are stored in secretory granules

and when activated, are transported quickly to the plasma membrane. P-selectin has a fast turnover on the surface and is quickly internalised. Sorting nexin-17 has a PX-domain which interacts with LDL-receptor family proteins NPxY motifs, enhancing endocytosis of P selectin and is found to be colocalised with early endosomal antigen-1 (EEA1) in early endosomes (Worby and Dixon, 2002). SNX17 regulates degradation of P-selectin by suppressing its entry into the inner membrane of multi-vesicular bodies. Multi-vesicular bodies budding from early endosomes act as transporters to late endosomes. Overexpression of SNX17 prevents the degradation of P-selectins by retarding the process which P selectins enter the inner membrane of multi-vesicular bodies transported to late endosomes for degradation (Knauf et al., 2005).

FERM-like domain containing nexins such as SNX17, SNX27 and SNX31 were proposed to interact with transmembrane proteins, including integrins such as α3, α5, α6 and β1-integrins. Sorting nexin-17 and nexin-27 bound to cytoplasmic domains of target proteins were able to direct these proteins away from the degradation process. Sorting nexin-17 has a PX-domain which binds to phosphotidylinositide-3-phosphate containing membranes in early endosomes, which are involved in the sorting of cargo proteins (Knauf et al., 2005, Steinberg et al., 2012). SNX17 was found to be bound to the membrane distal NPXY motif in β-integrin cytoplasmic tails and prevented the degradation of both the β and associated α subunits of the integrins. Cells blocked with bafilomycin (which inhibits endosomal acidification and blocks lysosomal degradation) are able to restore β1 integrins which are depleted in SNX17 null cells or cells treated with siRNA targeting SNX27. Depleting SNX17 led to degradation of β1 and β5 integrins in HeLa cells. Depletion of SNX17 also led to reduced focal adhesion area and adhesion size of fibroblasts. These cells also had increased speed and persistence in

directionality during cell migration as cells switched from α5β1 (which promotes cell stasis) to αVβ3 (which promotes cell migration) (Steinberg et al., 2012).

1.12. Sorting Nexin-17 and β1 integrins interaction

Nexin-17 has been found to compete with Kindlin binding to the β1 integrin cytoplasmic domain. When integrins are activated, Kindlinbinds to the cytoplasmic tail of integrin at the cell membrane (Brahme et al., 2012). As the integrins are internalised in early endosomes, Kindlin dissociates from the integrin cytoplasmic domain and the integrins may be degraded via the lysosomal pathway, as other membrane proteins. It is not known yet if the integrins remain in the highly activated state when internalised and how disassociation of kindlins from the cytoplasmic domain of β-integrin tail is mediated during the internalisation process (Brahme and Caldrwood, 2012, Böttcher et al., 2012, Steinberg et al., 2012). However, it was shown that Nexin-17 binding to this same site in the β1 integrin cytoplasmic domain in early endosomes prevents the degradation of the integrin and the integrin is recycled back to the surface instead of progressing to the protein degradation pathway (Böttcher et al., 2012, Steinberg et al., 2012, Brahme et al., 2012). This binding site shared by Kindlin and Nexin-17 in β1 integrins is homologous to a site in the β2 integrin, but the roles of these interactions in β2-integrin regulation are currently unclear.

1.13. Integrins and disease

Mutations in integrins and problems with regulation of integrin function could cause problems in the immune system, either resulting in immunodeficiencies due to lack of function in host defence system or a hyperactive immune system. Current therapies that target integrins aim to interfere with the binding of integrins with ligands or are aimed at the extracellular region of integrins. Integrin antagonists used in clinical studies can be humanized antibodies, synthetic peptides or non-peptide small molecules. An example of this includes antibodies that target α4β1 or α4β7 in treatment of multiple sclerosis (Abram and Lowell, 2009).

Here, we focussed on mutations that can occur in either β2 integrins or an associated α-integrin that could be involved with disease development.

1.13.1. Importance of β2 integrins in Leukocyte adhesion disorder

Leukocyte Adhesion Deficiency, (LAD) is a genetic syndrome associated with the loss of β2-integrin expression or function. It is a group of primary immunodeficiency disorders characterised by persistent infections, where immune cells are unable to adhere to endothelium and migrate to sites of inflammation or infected tissues. There are three different subtypes: LAD-I (effects in firm adhesion), LAD-II (defects in rolling) and LAD-III (defects in activation). Knock-out mice and patient studies have shown the importance of leukocyte adhesion in the host immune system. Most sufferers die early if not treated appropriately (Etzioni, 2009, Macpherson et al., 2013).

In the case of LAD-I, somatic mutations in the promoter region of the β2-integrin gene results in reduced or no expression of β2 integrin or mutations within the coding region led to truncated or non-functional β2 integrin (van de Vijver et al., 2012). LAD-II is caused by defects in fucosylation of selectin ligands and oligosaccharides such as PSGL-1. Mutation occurs in the gene for GDP-fucose specific transporter (SLC35C1) which affects its function and/or its subcellular localisation. As leukocytes required selectins on endothelial cells for rolling, this tethering process that activates LFA-1 and Mac-1 was affected during leukocyte migration to infected tissues (Helmus et al., 2006). In LAD-III, mutations were detected in the FERMT3 gene that encodes kindlin-3. Premature stop codons were introduced resulting in non-functional kindlin-3. As Kindin-3 binds to all β1, β2 and β3 integrins, defects in kindlin-3 are more complex than LAD-I (Moser et al., 2009). LAD-I and LAD-III patients display similar symptoms. In addition, LAD-III patients have Glanzmann-type bleeding disorder as kindlin-3 also regulates β3 integrin-mediated adhesion in platelets. Some patients also suffered from osteopetrosis where bones become harder and denser due to osteoclast-mediated bone resorption deficiency regulated by integrins (Malinin et al., 2009). Kindlin-3 is required for TCR-stimulated LFA-1 mediated adhesion in T cells, activation and spreading on dendritic cells. Loss of kindlin-3 affected NK cell activation and erythrocyte shape in LAD-III patients (Feigelson et al., 2011, Gruda et al., 2012).

1.13.2. Importance of R77H mutation in αM-integrin in Systemic Lupus Erythematosus patients

SLE (Systemic Lupus Erythematosus) is a Type III hypersensitivity disorder that affected many organs such as skin, lungs, liver, kidneys and the central nervous system which affect woman of the child bearing age (Liu and Davidson, 2012, Fagerholm et al., 2013). SLE predominantly affects women at a 9:1 ratio and is a clinically heterogenous autoimmune inflammatory disease with a strong genetic component and environmental components. It was estimated to affect 31 per 100,000 women in Europe, which is 50–75 % lower than in other populations and is more severe in non-European populations.

Large scale screening of single-nucleotide polymorphism (SNP) in patients suffering from this found that other than SNPs detected in the HLA region, there are a few SNPs associated in or near ITGAM gene which encodes for CD11b/αM-integrin which is involved in the formation of Mac-1 or Complement Receptor 3 (CR3) (Harley et al., 2008). The strongest association between SLE and ITGAM was rs1143679, with the odds ratio of 1.78 (Nath et al., 2008).

Mac-1 regulates leukocyte adhesion and emigration by interacting with various ligands such as ICAMs and fibrinogen and it is observed in SLE patients that neutrophils levels are increased. Mac-1 also has a role in clearance of immune complexes which is impaired in SLE patients and described as a disease mechanism (Nath et al., 2008). Inefficient phagocytosis by macrophages and neutrophils were suggested to lead to accumulation of apoptotic cells and cellular fragments, which could be presented by dendritic cells to T cells and may contribute to increased B cell activation, production of autoantibodies and finally further accumulation of immune complexes (Gaipl et al.,

2004). SLE patients appeared to have poor iC3b deposition of *Streptococcus pneumoniae* in their serum compared to healthy patients which could lead to poor opsonisation and clearance of the bacteria, increasing the susceptibility of these patients to bacterial infections. This poor iC3b deposition could also contribute to poor activation of complement activation pathway in clearance of immune complexes in SLE patients (Goldblatt et al., 2009).

Some SNPs in ITGAM have been found to affect the iC3b binding site, leading to impaired phagocytosis (Nath et al., 2008). The effects of rs1143679 were predicted using a protein crystal structure generated from the amino acid sequence and αVβ3 (Protein Data Bank: 1u8c) as a template. The polymorphism occurs within the **CD11b/αM-integrin** coding region and converts the normal arginine to histidine at position 77 of the **CD11b/αM-integrin p**olypeptide (R77H). This amino acid lies in the beta-propeller domain proximal to the ligand binding domain in the extracellular region of Mac-1 (Nath et al., 2008, Macpherson et al., 2013). This R77H polymorphism was suggested to be unlikely in affecting iC3b binding or influence interactions between the β-propeller domain and the βI domain. However, it was not possible to determine if the polymorphism would affect binding of other Mac-1 ligands using this model (Nath et al., 2008).

1.14. Purpose of Study

The main purpose of this study is to investigate the importance of the activation of β2-integrins and the regulation of β2-integrins in leukocytes. The questions that need to be answered include:

- What are the signalling pathways that regulate integrin activation and migration in lymphocytes?
- Are there differences in integrin regulation among lymphocyte groups?
- Is adhesion under static conditions differently regulated than under shear flow conditions?
- How are effector functions downstream of integrin activation and integrin-mediated adhesion regulated?

In order to answer these questions, the project is divided into three main approaches:

- Determining which AGC kinases regulate integrin-mediated adhesion in primary murine **B cells**
- Comparing the regulation of integrin-mediated adhesion in primary murine $CD8^+$ effector **cytotoxic T lymphocytes** to that in B cells
- Investigating the regulation of integrin-mediated adhesion leading to phagocytosis by focussing on two mutations associated with Leukocyte Adhesion Deficiency and Systemic Lupus Erythematosus: the triple threoninesT758-760 in the β2-integrin cytoplasmic domain and the R77H in the αM-integrin chain, respectively.

2. Materials and Methods

2.1. Materials

2.1.1. Mice

ADAP−/− mice were provided by Stefanie Kliche and were generated as described in (Kliche et al., 2006). The mice were bred and maintained under specific pathogen-free conditions in the Otto-von-Guericke University, Magdeburg, Germany, according to the guidelines of the State of Sachsen-Anhalt, Germany. PKCβ knock-out mice generated and provided by Michael Leitges as described in (Leitges et al., 1996), and approved by the Folkehelse Institute, Oslo (Norway) and performed according to its institutional guidelines and to the rules and regulations of the Federation of European Laboratory Animal Science Association´s (FELASA). PKD-null (PKD1/3 double knock-out) and PKD2-deficient mice were kind gift from Doreen Cantrell (University of Dundee) and were generated as described in Matthews et al., (2006). β2-integrin knock-out mice were donated by Colin Watts. The constitutive TTT/AAA β2-integrin knock-in mice were made on a C57Bl/6 background by TaconicArtemis and were described in Morrison et al., (2013). Wild-type C57/Bl6 mice were obtained from Charles River. 6-8 week old C57BL/6 wild-type mice were also donated by various group, eg.. John Foerster, Paul Crocker, Colin Henderson and Sharon Matthews (University of Dundee).

Mice were bred and maintained under specific pathogen-free conditions in the Wellcome Trust Biocentre at the University of Dundee in compliance with U.K. Home Office Animals (Scientific Procedures) Act 1986 guidelines. In all experiments, homozygous mice were used, with WT littermates as controls.

2.1.2. Cells

Murine A20 B cells (BALB/c B-cell lymphoma) were a gift from Sharon Matthews (Dundee, UK). The human T-cell lymphoma cell line clone Jβ2.7 which lacks CD11 chains, was a kind gift from N. Hogg (London, UK) and was generated as described in Weber et al., 1997. African Green Monkey kidney COS-1 cells were obtained from Jenny Woof (Dundee, UK). Murine brain endothelial bEnd.3 cells (ATCC® CRL-2299) were obtained from American type Culture Collections (ATCC). B cells were isolated from mouse spleens using Dynabeads® Mouse CD43 (Untouched™ B Cells), 114-22D, Invitrogen Life Technologies. $CD4^+$ were positively selected from whole cells from mouse spleen and lymph nodes using MACS CD4 (L3T4) MicroBeads for mouse 130-049-201 and $CD8^+$ T cells were positively selected from whole cells from mouse spleen and lymph nodes using MACS CD8a (LY-2) microbeads for mouse 130-049-401 from Miltenyi Biotec. The Active Rap1 pull-down kit was from Thermo Scientific. (Catalogue number 16120)

2.1.3. Reagents

All reagents used for cell culture were from Life Technologies/Invitrogen. RPMI 1640 (21875034) or DMEM (41966029) cell culture medium was supplemented with 10 % Fetal Bovine Serum (10500-064) and 1 % Penicillin-Streptomycin solution (15070063). Trypsin-EDTA was used to detach adhesive COS-1 cells (25300-054) and D-PBS (14190094) was used to rinse and detach bone marrow-derived dendritic cells or macrophages. All suspension cells (such as primary T cells and B cells), bEND.3 and COS-1 cells were grown in T25cm^2, T80cm^2 or T150cm^2 tissue culture flasks (Nunc) or

on 6—well Tissue culture plates (Nunc). Spleens were mashed and filtered through Cell culture strainer nylon mesh 70 μm filters (352350 BD Falcon). 90 mm non-tissue culture-treated plates (Greiner) or 6-well non-treated culture plates (351146, BD Falcon) were used for the culture of bone marrow-derived dendritic cells or macrophages.

In some experiments, cells were stimulated using phorbol esters, either Phorbol 12, 13-dibutyrate (PDBu) P1269-5MG or Phorbol 12-myristate 13-acetate (PMA), which were obtained from Sigma- Aldrich. To stimulate primary murine B cells through the B cell receptor, AffiniPure F(ab)'2 fragments Goat anti-mouse IgM, mu chain specific antibodies 115-006-020 from Jackson ImmunoResearch were used. To stimulate $CD4^+$ or $CD8^+$ T cells through the T cell receptor, the anti-CD3 monoclonal antibody (clone 2c11) MAB484 from R&D Systems was used. To stimulate A20 cells through the B cell receptor, cells were first treated with HM79-16 hamster anti-mouse Igβ mAb (a kind gift from Sharon Matthews) for 30 minutes and then stimulated using a Goat anti-armenian hamster IgG (H+L), (127-005-160, Jackson laboratories). To stimulate splenic T cells to develop into effector cytotoxic T lymphocytes, cells were stimulated with an anti-CD3 monoclonal antibody (clone 2c11) MAB484 from R&D Systems and IL-2 from R&D systems or Novartis ProLeukin IL-2. Recombinant Murine M-CSF (315-02, Peprotech) was added into DMEM cell culture medium to stimulate differentiation of bone marrow-derived macrophages. Recombinant Murine GM-CSF (315-03, Peprotech) was added into RPMI growth medium to stimulate differentiation of bone marrow-derived dendritic cells. To stimulate the expression of ICAM-1 and selectins on BEnd.3 cells, TNF-α from R&D systems was used. Quantities of reagents required are mentioned in relevant methods section.

Static adhesion assays were carried out using 96-wells Nunc Maxisorp plates, catalogue number 442404. Ligands to be immobilized onto the adhesion plates included fibronectin from human plasma (341635-1MG Calbiochem), mouse ICAM-1 (796-IC, R&D Systems), human ICAM-1 (ADP4-200, R&D Systems) and the human complement protein iC3b (204863-250UG, Merck). Ligands were reconstituted in PBS, except for fibronectin, which was reconstituted in RPMI for storage stability. Plates were blocked with 1 % milk in PBS (Marvel dried skimmed milk) for assays using murine primary cells, or 3 % Human Serum Albumin (A8763-5g, Sigma) for assays using Jurkat cells, or 1 % PVP Polyvinylpyrrolidone (P5288, Sigma) for assays using bone marrow-derived dendritic cells, to prevent unspecific binding. P-nitrophenyl phosphate disodium salt, hexahydrate, (4876-5gm, Merck) was used as a phosphatase substrate to detect adherent cells.

Adhesion assays under shear flow conditions were carried out using IBIDI u-Slide VI (0.4) uncoated plates, (IB-80601). ICAM-1 and CXCL12/SDF-1α (460-SD/CF, R&D systems) were immobilized in the channels. BEnd.3 cells were grown in IBIDI u-Slide VI (0.4) T/C-treated plates (IB-80606). Recombinant Mouse E-Selectin/CD62E Fc Chimera which was also co-immobilized with mouse ICAM-1 (575-ES-100) was from R&D Systems and it was reconstituted in PBS. 2D Migration assays were carried out using IB-80821 u-slide uncoated 8-well plates.

2.1.4. Buffers

10X PBS was made by dissolving 80g of NaCl, 2.0g of KCl, 14.4g of Na_2HPO_4, 2.4g of KH_2PO_4 in 800 ml of distilled water. The pH was adjusted to 7.4 before topping up with more distilled water to 1 litre. 1 part of this solution was diluted with 9 parts of water to make up PBS. To make up PBST (PBS with 0.05 % Tween-20), 0.5 ml of Tween-20 was added to 1 litre of PBS. TBST (TBS with 0.05 % Tween-20) was made with 50 mM Tris and 150 mM NaCl in distilled water. After the pH was adjusted to 7.6, 0.5 ml of Tween-20 was added to 1 litre of TBS.

ACK (Ammonium-Chloride-Potassium) lysis buffer to lyse red blood cells was made up of 0.15 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM EDTA, pH 7.3. The buffer was sterilised by autoclaving (All reagents were from Calbiochem or Sigma Aldrich.)

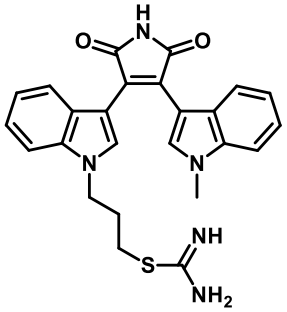
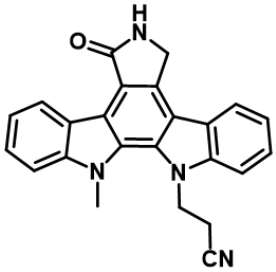
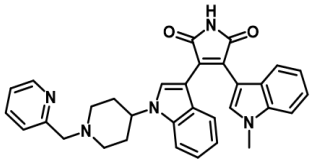
The high salt lysis buffer consisted of 350 mM sodium chloride, 50 mM Tris pH 7.4, 50 mM sodium fluoride, 10 mM EDTA, 1 % Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF and 40 μM β-glycerophosphate. One Complete Mini EDTA-free Protease Inhibitor Tablets (4693159001 Roche) was added to 10 ml of lysis buffer just before use to prevent degradation of proteins by enzymes in the cells during cell lysis.

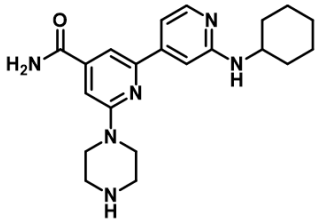
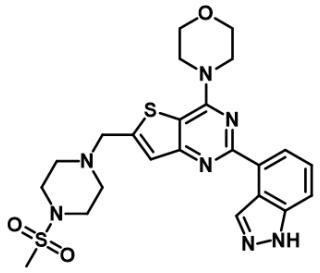
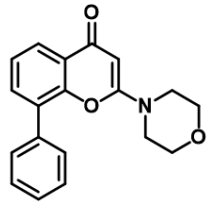
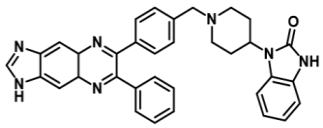
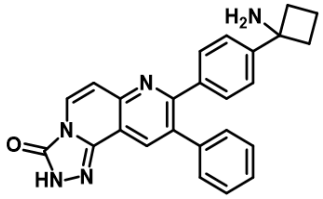
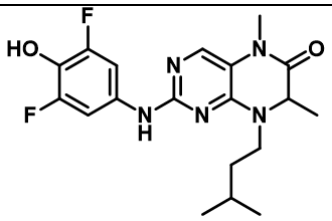
The low salt lysis buffer (for lysing macrophages) consisted of 150 mM sodium chloride, 50 mM Tris pH 7.4, 50 mM sodium fluoride, 10 mM EDTA, 1 % Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF, 40 μM β-glycerophosphate and 1 mM sodium vanadate. One Complete Mini EDTA-free Protease Inhibitor Tablets (4693159001 Roche) was added to 10 ml of lysis buffer just before use.

The adhesion medium was RPMI 1640 (life Technologies) or PBS supplemented with 40 mM Hepes (H4034, Sigma) and 0.1 % HSA (A8763-5g, Sigma) or 0.1 % BSA (A7906, Sigma)**.** All reagents were from Calbiochem or Sigma Aldrich unless otherwise stated.

The Gelatin Veronal Buffer which was used in phagocytosis assays consisted of 0.15 mM $CaCl_2$, 141 mM NaCl, 0.5 mM $MgCl_2$, 0.1 % gelatin, 1.8 mM sodium barbital and 3.1 mM barbituric acid, pH 7.3-7.4. The solution was aseptically filtered and stored at 4 °C.

2.1.5. Inhibitors

Inhibitors	Target	Company/ Catalogue Number	Conc. used	Structure	References
Ro-31-8220	PKC (classical PKCs: PKCα, PKCβ1, PKCβ2 and PKCγ, novel PKCε, bisindolymaleimide ATP competitive inhibitor	Merck Millipore/ Calbiochem 557521-500UG	5µM		(Wilkinson et al., 1993, Powell et al., 2003, Spitaler and Cantrell 2004)
Gö6976	PKC (classical PKCs:PKCα, PKCβ1 and atypical PKCµ), ATP-competitive inhibitor	Merck Millipore/ Calbiochem 365253	10µM		(Martiny-Baron et al., 1993, Gschwendt, et.al, 1996)
Enzastaurin	PKCβ, bisindolymaleimide ATP competitive inhibitor	Selleck Chemicals S1055	5µM		(Graff et a., 2005, Querfield et al., 2006)

Novartis 12a	PKD (PKD1, PKD2, PKD3)	DSTT unit College of Life Sciences, University of Dundee	5μM		(Monovich et al., 2010, Meredith et al., 2010)
GDC-0941	PI3K, ATP-competitive inhibitors	Selleck Chemicals S1065	1μM		(Folkes et al., 2008)
LY294002	PI3K, ATP-competitive inhibitors	Merck Millipore/ Calbiochem 440204-1MG	50μM		(Vlahos et.al., 1993)
Akt VIII inhibitor (Akti-1/2 isozyme selective)	Akt (target Akt Pleckstrin Homology domain, allosteric inhibitor)	Merck Millipore/ Calbiochem 124017	10μM		(Logie et al., 2007)
MK2206	Akt (target Akt Pleckstrin Homology domain, allosteric inhibitor)	Selleck Chemicals S1078	5μM		(Li et al., 2009)
BI-D1870	RSK(ATP-competitive inhibitor of the N-terminal AGC kinase domain of RSK)	Symansis SY-BI-D1870	20μM		(Sapkota et al., 2007)

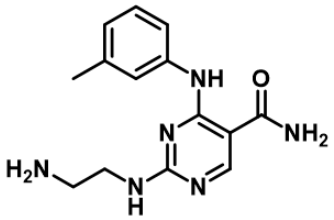
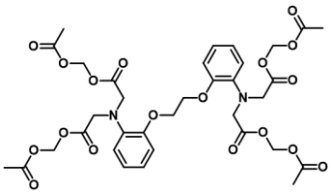
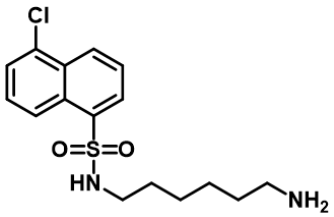
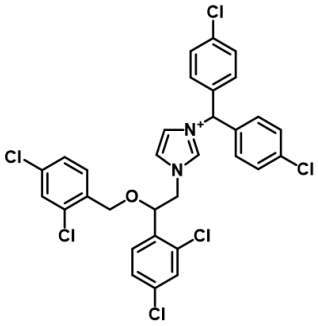
Syk II inhibitor	Syk (ATP-competitive inhibitor of Syk)	Merck Millipore/ Calbiochem	10uM		(Hisamichi et al., 2005)
Bapta-AM	Calcium (cell-permeant chelator more selective for calcium than magnesium)	Life Technologies	30uM		(Bird et al., 2008)
W7	Calcium (Calmodulin Antagonist)	Merck Millipore/ Calbiochem 681629-10MG	50µM		(Hidaka et al., 1981)
Calmidazolium	Calcium (Calmodulin inhibitor and calcium channel inhibitor)	Merck Millipore/ Calbiochem 208665-10MG	5 µM		(Gietzen et al., 1982)

Table 3. Inhibitors used in this study.

In addition, the proteasome inhibitor MG-132 (474791-5MG, Merck Millipore Calbiochem), the lysosome inhibitor bafilomycin (B1793, Sigma) and endosomal inhibitor primaquine (160393, Sigma) were used in some experiments. All inhibitors were dissolved in DMSO (D2650-100ml, Sigma).

2.1.6. Reagents for Western Blot

NuPAGE LDS Sample Buffer (NP0007) and NuPAGE Reducing Agent (NP0004) were used in the preparation of protein samples for SDS-PAGE Electrophoresis. Pre-cast polyacrylamide gels used were NuPAGE Novex 4-12 % Bis-Tris Gel 1.0 mm with 10-wells (NP0321Box) or NuPage Novex 4-12 % Bis-Tris Gel 1.0 mm with 12-wells (NP0322Box). The protein standard used was Pre-stained SeeBlue Plus 2 (LC5925). Electrophoresis Running buffers was either NuPAGE MOPS (NP0001) or MES Running Buffer (NP0002), supplemented with NuPAGE Antioxidant NP0005. SDS-PAGE Electrophoresis was performed using the XCell SureLock Mini-Cell Blot Module. All reagents and equipment used for Western blotting were from Life technologies/Invitrogen. Western blot transfer was performed using NuPage Transfer buffer (NP00061) and the blot was transferred onto Protran BA85 nitrocellulose membranes, pore size 0.45 μm (Z613630-1EA, Sigma). Ponceau S Stain (Sigma P7170) was used to check transfer efficiency. Amersham ECL Prime Western Blotting Detection Reagent (RPN2133/ RPN2236) was used for detection. Fuji Medical Super RX films were developed using a Kodak Imaging developer.

Antibody Type	Source	Company	Catalogue number	Western Blot dilution	Detection Size (kDa)
alpha-actinin	Mouse monoclonal	Millipore (Chemicon)	MAB1682	1:1000 3% milk	100
Cofilin	Rabbit polyclonal	Cell Signalling	#3312	1:1000 5% BSA	19
Filamin A Antibody, clone PM6/317	Mouse monoclonal	Millipore (Chemicon)	MAB1678	1:5000 3% milk	280
kindlin-3	Rabbit polyclonal	Sigma Aldrich	SAB 4200013	1:1000 3% milk	72
nexin-17	Rabbit polyclonal	Proteintech	10275-1-AP	1:1000 3% milk	53
nexin-3	Rabbit polyclonal	Abcam	ab56078	1:1000 3% milk	19
p-Akt (Ser473)	Rabbit polyclonal	Cell Signalling	#9271	1:1000 5% BSA	60
p-Akt (Thr308)	Rabbit polyclonal	Cell Signalling	#9275	1:1000 5% BSA	60
p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10)	Mouse monoclonal	Cell Signalling	#9106	1:2000 5% milk	42,44
p-PKD / PKCμ (Ser916)	Rabbit polyclonal	Cell Signalling	#2051	1:1000 3% milk	115
PKCbeta	Mouse monoclonal	BD Biosciences	610127	1:500 3% milk	80
PKD 1/2 (PKCμ)	Rabbit polyclonal	Cell Signalling	#2052	1:1000 3% milk	115
p-myosin Ser19	Rabbit polyclonal	Cell Signalling	#3671	1:1000 5% BSA	18
p-myosin, Ser18/19	Rabbit polyclonal	Cell Signalling	#3674	1:1000 5% BSA	18

p-p38 MAPK (Thr180/Tyr182)	Mouse monoclonal	Cell Signalling	#9216	1:2000 5% milk	43
p-Pyk2 (Tyr402)	Rabbit polyclonal	Cell Signalling	#3291	1:1000 5% BSA	116
p-Zap-70 (Tyr319)/ p-Syk (Tyr352)	Rabbit polyclonal	Cell Signalling	#2701	1:1000 5% BSA	70 (Zap-70), 72 (Syk)
Rap1	Rabbit polyclonal	Pierce	1862344 (Part of 16120)	1:1000 5% BSA	24
Src	Rabbit polyclonal	Cell Signalling	#2108	1:1000 5% BSA	60
talin 8D4	Mouse monoclonal	Santa Cruz	sc-59881	1:5000 3% milk	230
Total Akt	Rabbit polyclonal	Cell Signalling	#9272	1:1000 5% BSA	60
Total myosin	Rabbit polyclonal	Cell Signalling	#3672	1:1000 5% BSA	18
β2-integrin C71/16	Rat monoclonal	Abcam	ab19580	1:1000 3% milk	~80
Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate	Goat polyclonal secondary	Pierce	31460	1:10000 3% milk	-
Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP conjugate	Rabbit polyclonal secondary	Pierce	31450	1:5000 3% milk	-
Goat anti-Rat IgM Secondary Antibody, HRP conjugate	Goat polyclonal secondary	Pierce	31476	1:5000 3% milk	-

Table 4. Antibodies used in western blotting

All antibodies were diluted in either milk or BSA in PBST, except for myosin antibodies which were in TBST.

2.1.7. Flow cytometry Antibodies

Antibody Type	Source	Company	Catalogue number	Conc.	Conjugate
B220 (RA3-6B2)	Rat monoclonal	EBioscience	11-0452-82	1:100	FITC
CD19 (1D3)	Rat monoclonal	BD Biosciences	557655	1:200	APC-Cy7
CD4 (L3T4 RM4-5)	Rat monoclonal	BD Bioscience	553052	1:200	PerCp Cy5.5
CD8a (53-6.7)	Rat monoclonal	BD Bioscience	557654	1:200	APC-Cy7
FC-block (CD16/CD32 4.4G2)	Rat monoclonal	BD Biosciences	553142	1:70	-
F4/80 (BM8)	Rat monoclonal	EBioscience	47-4801-80	1:200	APC-Cy7
CD11a (2D7)	Rat monoclonal	BD Biosciences	553121	1:200	PE
CD11b (M1/70)	Rat monoclonal	BD Biosciences	553312	1:200	APC
CD11c (HL7)	Armenian hamster monoclonal	BD Biosciences	558079	1:200	PE-Cy7
CD18 (C71/16)	Rat monoclonal	BD Biosciences	553292	1:100	FITC
CD11b	Mouse monoclonal	BD Biosciences	333142	1:200	PE
CD18	Mouse monoclonal	BD Biosciences	347953	1:100	FITC

Table 5. Antibodies used in Flow Cytometry.

Live/Dead staining of cells were done using Live/Dead® AmCyan kit L34957 from Molecular Probes or DAPI (Calbiochem) at 5μM in final solution.

2.2. Methods

2.2.1. Cell culture of cell lines

Freezing cell lines

Early passage (passage 2-5) cultures of cells were passaged the day before freezing. Adherent cells were detached and suspension cells were pooled by centrifugation at 1200rpm (300g) for 5 minutes using Heraeus Labofuge 400, Thermo Scientific. The cells were resuspended at a density of 2-5x10^6/ml in 10 % DMSO/FBS. Aliquots of 1 ml of cell suspension per cryovial were then placed in a Mr. Frosty™ Freezing Container (Thermo Scientific), which was filled with Methanol (Merck Millipore). The container was placed in -80 °C overnight, prior to transferring the cryovials into liquid nitrogen tanks for long term storage.

Thawing of cell lines

A cryovial of frozen cells was quickly removed from liquid nitrogen storage and placed immediately into a 37 °C water bath for about 1 minute with gentle swirling. Once the vial was about 80 % defrosted, the cell suspension in the cryovial was resuspended a few times slowly with a pipette before transferring the cell suspension into a T25cm^2 flask containing 5 ml of pre-warmed cell medium. The growth of cells was monitored the next day and another 5 ml of medium was added into the tissue culture flask. Cells were monitored daily until the recommended cell density was reached.

Maintaining A20 B cells

Murine A20 B cells (BALB/c B-cell lymphoma) were cultivated in RPMI 1640 medium supplemented with 10 % fetal calf serum, 1% or 10 U/ml penicillin-streptomycin, 2 mM glutamine, and 50 μM 2-β-mercaptoethanol at 37 °C in 5 % CO_2. These cells were passaged every 2-3 days and maintained at a cell density of $0.2x10^6$ to $1.0x10^6$/ml at all times.

Maintaining untransfected and transfected Jurkat Jβ2.7 cells

Jurkat Jβ2.7 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% or 10 U/ml penicillin-streptomycin, 2 mM glutamine, with G418 (0.4 μg/ml) (G1279-iG, Sigma) to maintain stably transfected cell populations at 37 °C in 5 % CO_2. These cells were passaged every 3 days and maintained at a cell density of $0.2x10^6$ to $2.0x10^6$/ml at all times.

Maintaining BEnd.3 and COS-1 cells

Stocks of cells were grown from American type Culture Collections (ATCC), (ATCC® CRL-2299) until ready for use in experiments. Cells were maintained in DMEM medium (with 10% FBS and 1% Penicillin-Streptomycin solution) in T75cm^2 flask. The cells were passaged every 3-5 days (when the culture reached 90 % confluence). To detach cells, medium was removed with a pipette and the cells were rinsed with 10 ml of D-PBS. 2.5 ml of Trypsin-EDTA was added into the flask with

gentle swirling to cover the surfaces and the flask was incubated at 37 °C in 5 % CO_2 for 1-2 minutes. The flask of cells was tapped against the palm of a hand a few times to aid the detachment of cells. 7.5 ml of DMEM medium was then added into the flask and the cells were pipetted up and down a few times to ensure the cell layer was evenly broken up. The cells were split at 1:5 or 1:4 for maintenance or 1:2 for use in experiments the next day.

Maintaining BEnd.3 for use in shear flow assays

The cells were passaged at 1:2 the day before seeding the cells into IBIDI μ-slide VI (0.4) T/C treated plates for experiments. Cells were seeded into μ-slides by pipetting 30μl of cells at $0.5x10^6$cells/ml into the channel and letting the cells adhere for 30 minutes in the incubator at 37 °C, 5 % CO_2. Reservoirs at the end of the channels were then filled 200 μl of medium and the slides were incubated overnight, with frequent medium changes every 8-12 hours over the next 48 hours/ 18 hours. Before the experiment, the existing medium was replaced with DMEM medium containing 10 ng/ml TNF-α and incubated in the same conditions with frequent medium changes to stimulate expression of P-selectins, E-selectins and ICAM-1.

Alternatively, to obtain polarised cell layers, μ-slides were connected to the IBIDI pump system set up in the incubator according to manufacturer's instructions after the cells were seeded and the reservoirs filled. The cells were allowed to grow in the presence of continuous unidirectional flow at 2 dynes/cm^2 for the first 30 minutes, 5 dynes/cm^2 for the next 30 minutes and then 15 dynes/cm^2 for the next 3 days. 10 ng/ml TNF-alpha was added into the medium in the pump system 18 hours prior to the experiment.

2.2.2. Isolation and culture of primary cells from mice

Isolation of splenocytes

Spleens extracted from mice were mashed in cell medium (RPMI 1640 medium supplemented with 10 % fetal calf serum, 1 % or 10 U/ml penicillin-streptomycin, 2 mM glutamine, and 50 μM 2-β-mercaptoethanol) with a 2-ml syringe handle and then passed through a 70 μm filter. The cell suspension was centrifuged at 300 g for 5 minutes (using Heraeus Labofuge 400, Thermo Scientific) and the supernatant was removed. The cell pellet from each spleen was resuspended into 5 ml ACK (Ammonium-Chloride-Potassium) lysis buffer (containing 0.15 M NH4Cl, 0.01 M KHCO3 and 0.1 mM EDTA) to lyse red blood cells by incubating the cells at room temperature for a minimum of 3 minutes to a maximum of 5 minutes. 45 ml of cell culture medium was then added to the tube and centrifuged as before to remove the mixture of ACK lysis buffer and cell medium. The remaining cells, consisting of mainly B and T lymphocytes and monocytes, were then resuspended in 6ml of cell medium and transferred into a 6-well tissue culture plate at 37 °C in 5 % CO_2 for 30 minutes to allow adherent monocytes to settle. The plates were then gently swirled and the remaining cell suspension (predominantly B cells) was used in experiments.

Isolation of B cells from mouse spleens

Resting B cells were purified from splenocyte suspensions using Untouched CD43 Dynabeads (Life Technologies/Invitrogen) according to the manufacturer's instructions. T cells, monocytes/macrophages, granulocytes, activated B cells, plasma cells, dendritic

cells and Natural Killer Cells express CD43 cells on their surface and therefore bind to these magnetic beads. Resting B cells do not express CD43 and therefore do not bind to these magnetic beads. Beads and attached cells can therefore be pelleted using a magnet, allowing easy separation of B cells from other types of cells.

Splenocytes were obtained as described earlier and resuspended in cell medium (RPMI 1640 medium supplemented with 10 % fetal calf serum, 1 % or 10U/ml penicillin-streptomycin, 2 mM glutamine, and 50 μM 2-β-mercaptoethanol). Cells were washed once with PBS and suspended in Isolation buffer (containing 0.1 % BSA and 2 mM EDTA) at a cell density of 5 $x10^7$/ml in 15-ml centrifuge tubes. At the same time, another set of tubes containing the required Dynabeads was prepared, containing 125 μl of Dynabeads per every 5 $x10^7$/ml of cells. The beads in the bottle were mixed by vortexing and transferred out using a P200 pipette tip, with the narrow end of the tip cut off to allow for a bigger opening so as not to destroy the beads. To wash the beads, 1 ml of isolation buffer was added to the beads suspension and mixed by vortexing, and the mixture was then placed onto a Dynal magnet rack (Life Technologies/Invitrogen) for 1 minute to concentrate the beads into a pellet, so that the remaining wash buffer could be removed with a pipette. Once the supernatant was removed, 125 μl of Isolation buffer per 125 μl beads was added into the tubes. The tubes were removed from the rack and the beads were mixed by vortexing. Splenocytes in Isolation buffer were then added into the tubes containing the beads and placed on a rotator for gentle mixing for 20 minutes at room temperature. At the end of the incubation period, the cells were mixed by gentle pipetting with a P1000 tip 10 times and 2 ml of Isolation buffer was added with further mixing. The tubes were placed onto the Dynal magnet for 2 minutes to concentrate the beads and attached CD43-expressing cells nto a pellet. The supernatant containing the B cells was then removed and transferred to a new 15-ml tube that was

placed on ice. Another 2 ml of Isolation buffer was added to the beads with mixing and was left to settle using the magnet, as stated in the previous step, to extract more B cells. Purified B cells were counted and suspended in adhesion medium to be used immediately in experiments. The extraction procedure yielded ~98 % B cells positive for CD19 (a B cell marker), as analysed by flow cytometry.

Isolation of $CD4^+$ or $CD8^+$ T cells

$CD4^+$ T cells were purified using MACS CD4 (L3T4) MicroBeads for mouse (130-049-201, Miltenyi) while $CD8^+$ T cells were purified using MACS CD8a (LY-2) Microbeads for mouse (130-049-401, Miltenyi). Lymph nodes (Inguinal, Mesenteric, Axilary and Branchial) were removed from the mouse and placed in 2 ml cell medium (RPMI 1640 medium supplemented with 10 % fetal calf serum, 1 % or 10 U/ml penicillin-streptomycin, 2 mM glutamine, and 50 μM 2-β-mercaptoethanol) in a 6-well plate and these were passed through a 70 μm filter using a 2-ml syringe handle. Splenocytes were obtained as described earlier and combined with the cells from lymph nodes and then passed through a 30 μM filter to remove cell clumps.

The cells were incubated with CD4 (L3T4) MicroBeads to isolate $CD4^+$ cells, while CD8 (LY-2) MicroBeads were used to isolate $CD8^+$ cells. The isolation was carried out on ice or at 4 °C at all times to prevent unspecific binding. The cell suspension was centrifuged at 300 g for 10 minutes at 4°C using Eppendorf Microcentrifuge 5415R/5415D and the supernatant was removed. The magnetic beads were mixed by vortexing and P200 pipette tips, with the narrow ends cut off, were used to transfer the beads. The cells were suspended in 90 μL of RPMI containing 5 % FBS per 10^7 total

cells and 10 µL of CD4 (L3T4) MicroBeads or CD8 (LY-2) MicroBeads per 10^7 total cells was added to the cell suspension. The cells were mixed well by vortex and incubated at 4−8 °C for 30 minutes to magnetically label the cells. The cells were washed using 5 ml of RPMI containing 5 % FBS. The sample was centrifuged at 300 g for 5 minutes and the cells were resuspended in 1 ml of RPMI containing 2 % FBS.

A MACS® column LS Columns (130-042-401) was placed in the magnetic field of a QuadroMACS Separator (130-090-976) attached to an ACS MultiStand (130-042-303). The LS column was prepared by rinsing with 3 ml of RPMI containing 2 % FBS. To separate the $CD4^+$ or $CD8^+$ labelled cells from the cell suspension, the cell suspension was loaded onto LS Columns. The magnetically labelled $CD4^+$ cells or $CD8^+$ cells are retained on the column while the unlabeled cells pass through. The column was rinsed thrice using 3 ml of RPMI containing 2 % FBS, each time once the column reservoir was empty. The LS column was then removed from the magnetic field/separator and placed in a 50 ml tube. The magnetically retained $CD4^+$ cells or $CD8^+$ cells were eluted as the positively selected cell fraction, by adding 5 ml of RPMI containing 2 % FBS and the magnetically labelled cells were immediately flushed out by firmly applying the plunger supplied with the column. The labelled fraction of cells was passed through another new LS column to improve purity and finally resuspended in adhesion assay medium for other assays. Cells were of 95-98 % purity, as determined by flow cytometry. (Data not shown.) The eluted cells were used for adhesion assays as soon as possible.

2.2.3. Cell culture of primary cells from mice

Generation of effector cytotoxic T lymphocytes

Effector cells were defined as activated T cells that expressed CD3 (part of the T cell receptor), CD4 (for Helper T cells) or CD8 (for cytotoxic T cells) and CD25 (the IL-2 receptor), and had low expression of CD62L and high expression of CD44. To generate effector cytotoxic T lymphocytes, splenocytes from wild-type, β2 integrin−/−, or ADAP−/− mice were obtained as described previously, except that monocytes were not removed. Splenocytes were activated for 2 days with 0.5 μg/ml anti-CD3 together with 20 ng/ml IL-2 in cell medium (RPMI 1640 medium supplemented with 10 % fetal calf serum, 1 % or 10 U/ml penicillin-streptomycin, 2 mM glutamine, and 50 μM 2-β-mercaptoethanol) and in 37 °C in 5 % CO_2. Cells were then washed and thereafter maintained in cell medium supplemented with 20 ng/ml IL-2. Cells were maintained at a cell density of at least 10^6 cells/ml for another 4–5 days at 37 °C in 5 % CO_2 and thereafter used for experimental purposes between Days 7-10 since culture. Effector cytotoxic T lymphocytes generated this way contained approximately 90% $CD8^+$ and approximately 10% $CD4^+$ when detected using flow cytometry, and were $CD62L^{low}$, $CD44^{high}$. (Data not shown. Experiments done by Vicky Morrison.)

Culturing bone marrow-derived dendritic cells

Back leg bones (Femur and Tibia) and upper arm humerus from 1 mouse were used to generate bone marrow-derived dendritic cells. The bone marrow was flushed out using a 2 ml syringe and a 25 G needle filled with RPMI medium supplemented with 10 % FBS

and 1 % pen-strep solution. Cell aggregates were broken up using a P1000 pipette and the cells were suspended in 2 ml medium. Cells were counted and suspended at 10^7 cells/ml. 10 ml medium supplemented with 10 ng/ml GM-CSF was dispensed into each of 10 non-treated 10 cm petri-dishes and 200 ul of bone marrow cell suspension was added into the middle of each plate without mixing. The plates were left undisturbed in 37 °C, 5 % CO_2 incubator for 3 days.

After 3 days, 5ml of warm medium supplemented with 10 ng/ml GM-CSF was slowly added to the sides of each plate. Another 3 days later, 9 ml of medium from each plate was gently removed ensuring that the cells were not disturbed or removed. Cells from TTT/AAA-β2-integrin knock-in mice tended to be less adhesive and therefore the medium removed during the culture procedure was centrifuged at 300 g for 5 minutes (using Heraeus Labofuge 400) and the cells were added back to the plates with fresh medium. 10ml of medium supplemented with GM-CSF was added gently by slowly pipetting down the sides of each plate. This medium change procedure was repeated 3 days later. At the end of the 10 day culture, bone marrow-derived dendritic cells were ready to be harvested. Depending on experiments, cells were treated with fresh medium with 10 ng/ml GM-CSF 24 hours before harvest and reseeded onto non-tissue culture-treated 6-well plates at 1-2 million cells per well if necessary. To harvest cells from petri-dishes, the medium was collected and 5ml of aseptically filtered 4 mM EDTA in PBS was added to the plates and incubated for less than 10 minutes at 37 °C to detach the rest of the cells. Cells were detached from the plates by repeated pipetting 10-20 times and by rinsing the plates with additional RPMI medium. All the collected cells were then centrifuged at 300 g for 5 minutes (using Heraeus Labofuge 400) to remove the medium and EDTA and suspended in fresh medium with GM-CSF until used in experiments. Cells were used without maturation step.

Culturing murine bone marrow-derived macrophages

Arm bones (Humerus) and Back leg bones (Femur and Tibia) from 1 mouse used for macrophage culture. The bone marrow was flushed out using a 2 ml syringe and a 25G needle filled with DMEM medium supplemented with 10 % FBS and 1 % Penicillin-Streptomycin solution. Cell aggregates were broken up using a P1000 pipette and the cells were suspended in 2ml medium. Cells were counted and suspended in medium at 10^7cells/ml. 9ml of medium supplemented with 10ng/ml of M-CSF was dispensed into a sterile non-treated 10cm petri-dish and 1ml of bone marrow cell suspension was added into the middle of each plate without mixing. The plates were left undisturbed in 37 °C, 5 % CO_2 incubator for 3 days. After 3 days, 2 x 5 ml of warm medium supplemented with 10ng/ml M-CSF was slowly added to the sides of each plate. After another 3 days, 9ml of medium was gently removed from the side of the plates and centrifuged to retain cells. 10ml of fresh medium with M-CSF was replaced back into the plates, together with the cells that were removed during the change of medium. At the end of 7 days since the start of the culture of bone marrow cells, bone marrow-derived macrophages were ready to be harvested. Depending on experiments, cells were treated with fresh medium with 10 ng/ml M-CSF and reseeded onto non-tissue culture treated 6-well plates at 1-2 million cells per well 24 hours before harvest. To harvest cells, 20 ml of medium was collected and 5ml of aseptically filtered 4 mM EDTA in PBS was added to the plates and incubated for less than 10 minutes at 37 °C to detach the remaining cells. Cells were detached from the plates by repeated pipetting 10-20 times and by rinsing the plates with additional medium. All of the collected cells were centrifuged at 300 g for 5 minutes (using Heraeus Labofuge 400) to remove the EDTA containing medium.

2.2.4. Pre-treatment of cells with inhibitors

Cells were counted and washed by centrifugation, then resuspended in adhesion assay medium at a cell density of $1x10^6$/ml and left to rest at 37 °C with 5% CO_2 for 15 minutes. The cells were then transferred into T25cm^2 flasks where stock inhibitors dissolved in DMSO were added at 1:1000 dilution at base of the flask when tilted slightly. The volume of DMSO added was limited to 50 µl per 10 ml if possible and an equivalent amount of DMSO without inhibitors was added into control flask of cells. All the cells were swirled gently to mix and returned to the incubator at 37 °C with 5 % CO_2 for 30 minutes to 60 minutes for the inhibitor treatment.

2.2.5. Static adhesion assays

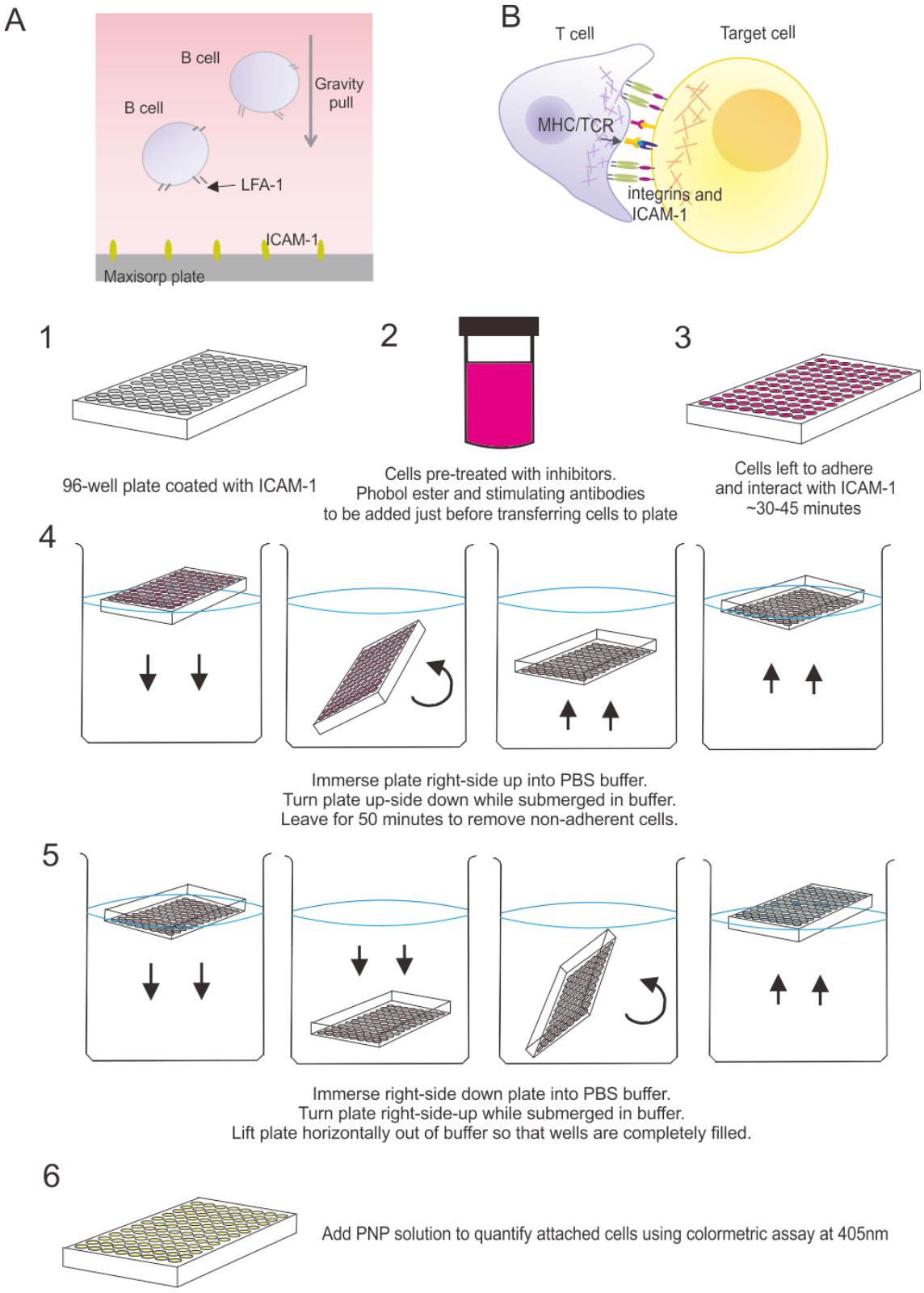


Figure 10. Schematic summary of static adhesion assay.

(A) Graphic representation of static adhesion assay set-up used to mimic (B) cell-cell interaction during cell adhesion process between two cells such as during T cell interacting with target cell. (1-4) Summary of the static adhesion assay in graphic form.

100 µl of integrin ligands Fibronectin (10 µg/ml), ICAM-1 (6 µg/ml) or iC3b (6 µg/ml) was added in duplicate/triplicate into flat-bottom 96-well Maxisorp plates (Nunc) by overnight incubation at 4 ^{o}C. Non-adhered ligands were removed by pipetting and the wells were blocked with 1 % milk/PBS (for assays using primary mouse lymphocytes) or 3 % HSA (for assays using Jurkat cells) or 1 % PVP (for assays using bone marrow-derived dendritic cells) for 1 hour 15 minutes at 37 ^{o}C. Control wells without any ligands were set aside and also blocked with 1 % milk/PBS or 3 % HSA or 1 % PVP to block unspecific binding to plastic. The wells were rinsed twice with 200 µl of PBS to remove excess blocking solution and left with 200 µl of PBS in the wells and the plate was stored on ice until use. The PBS was removed from the wells by pipetting immediately before adding the cells to the plate as described below.

Cells that had been pre-treated with inhibitors in cell adhesion medium as mentioned earlier were transferred into 15ml tubes on ice and mixed well to resuspend the cells. Stimulants were added to the cells in the tubes immediately before transferring the cells onto the 96-well plates with immobolized ligands. Cells were stimulated using 200 nM phobol ester (PDBu), 10 µg/mL stimulatory anti-BCR antibodies or 10 µg/ml anti-CD3. The tubes were inverted a few times to mix well and 400 µl of the cell suspension was transferred onto the wells with or without immobilized ligands.

A separate total plate was set up simultaneously to measure absorbance of total cells added per well. 200 µl of non-treated cell suspension was pipetted into a 1.5 ml tube

and washed with PBS once by centrifugating at 10,000 g for 30 seconds. The cells were resuspended in 200 µl PBS and 50 µl of the cell suspension of non-treated cells were added to each well in triplicate. Control wells with PBS without cells were also set up on this plate.

The cells in plates with immobilized ligands were allowed to adhere for 30 minutes (45 minutes for anti-CD3 stimulation or 20 minutes for dendritic cells) in a 37 °C incubator with 5 % CO_2. Unbound cells were then removed by gently and slowly immersing the plates in 2L of PBS with 2 mM $Mg2^+$ in a 4 L beaker, and then inverting the plates to enable it to float at the surface of the liquid for 50 minutes at room temperature. During this gentle washing step, unbound cells fall out of the plates due to gravity. The plates were then immersed again in PBS buffer and turned right side up slowly to ensure all wells were still filled to the brim with buffer when removed from the beaker. 350 µl of buffer was removed from each well, leaving 50 µl buffer with bound cells in the wells. The bound cells were lysed with 100 µl of PNP/lysis solution per well, e.g. 1 % Triton-X-100 (Sigma), 50 mM sodium acetate (Sigma), pH 5 containing 3 mg/ml p-nitrophenylphosphate (Calbiochem). The PNP solution was also added into the wells of the control (total) plate. All the plates were incubated for 1 hour at 37 ºC in the dark. The reaction was terminated with 1 M NaOH (Calbiochem) and the absorbance at 405 nm was measured and using BioTek ELx808 plate reader.

After obtaining absorbance readings of all wells, the average background value from "only PBS-containing wells" were first deducted off all other wells (total cells and experimental wells). To calculate values for "total cells" (400µl cell suspension): Readings from the "total cells plate" wells were multiplied by 8 as only 50µl cell suspension was used per well on the total plate to prevent out of range absorbance

readings (vs 400µl of cell suspension on adhesion plates). Then, readings from wells on the adhesion plate (after deducting PBS background readings) were expressed as a percentage of the total cell absorbance values.

To express data as "fold-change", average readings from control wells with unstimulated, non-inhibitor treated cells adhered to immobilised ligands are considered as "1". Readings from wells containing stimulated and/or inhibitor-treated cells adhered to immobilised ligands are expressed as fold-change by dividing the values from such wells by values from control wells.

2.2.6. Shear flow adhesion assays

The required ligands were immobilized onto Ibidi µ-slides VI 0.4, for example at 6µg/ml or as described in the Results section, and left overnight at 4°C. For assays assessing cell adhesion to endothelial cells, bEnd.3 cells were seeded onto plates at a concentration of $0.5x10^6$ cells/ml 48 hours before the experiment. TNF-α at a concentration of 10ng/ml was added into the growth medium 18h before the experiment to stimulate ICAM-1 expression on bEnd.3 cells. Where stimulation of cells required SDF-1 instead of phobol ester or antibodies specific to cell surface receptors, SDF-1 (5 µg/ml) was pipetted into the channels with immobilized ligands or layer of bEnd.3 cells for a further 30 minutes at 37 ^oC in an incubator with 5 % CO_2.

Leukocytes were suspended at a density of $1x10^6$ cells/ml in adhesion assay medium (RPMI with 0.1 %BSA, 40 mM Hepes and 2 mM $MgCl_2$), or PBS binding medium (supplemented with 0.1%BSA, 40 mM Hepes and 2 mM $MgCl_2$) for 15 minutes and at

least 5-10ml of cell suspensions were prepared for each run in 50 ml tubes. T cells were pretreated with inhibitors for 30 minutes or 1hour (depending on the inhibitor) at 37 ^{o}C incubator with 5 % CO_2. To stimulate the cells, the tubes were inverted a few times to resuspend the cells before and after addition of the cell stimulants. The cells were then incubated with 200 nM PdBu for 3 minutes, 10 μg/ml anti-BCR antibodies for 5 minutes at 37 °C for B cells or anti-TCR antibodies for 5 minutes. The cell suspension was then injected into a flow system that used a silicone tubing loop (IBIDI white perfusion set, length 50cm. Internal Diameter of 0.8mm) connected to a Multi-phaser NE-1000 syringe pump (New Era Pump Systems Inc.), allowing the cells to flow at a continuous shear flow rate of 0.3 dynes/cm^2. Other shear rates such as 0.2 dynes/cm^2, 0.5 dynes/cm^2, 0.8 dynes/cm^2, 1 dyne/cm^2, 1.2 dynes/cm^2 and 2dynes/cm^2 were also used by adjusting the volume of medium dispensed per minute. Cells were monitored by microscopy over a 10 minute period and the number of adhered cells determined in the field of view at 1 minute intervals by manual counting.

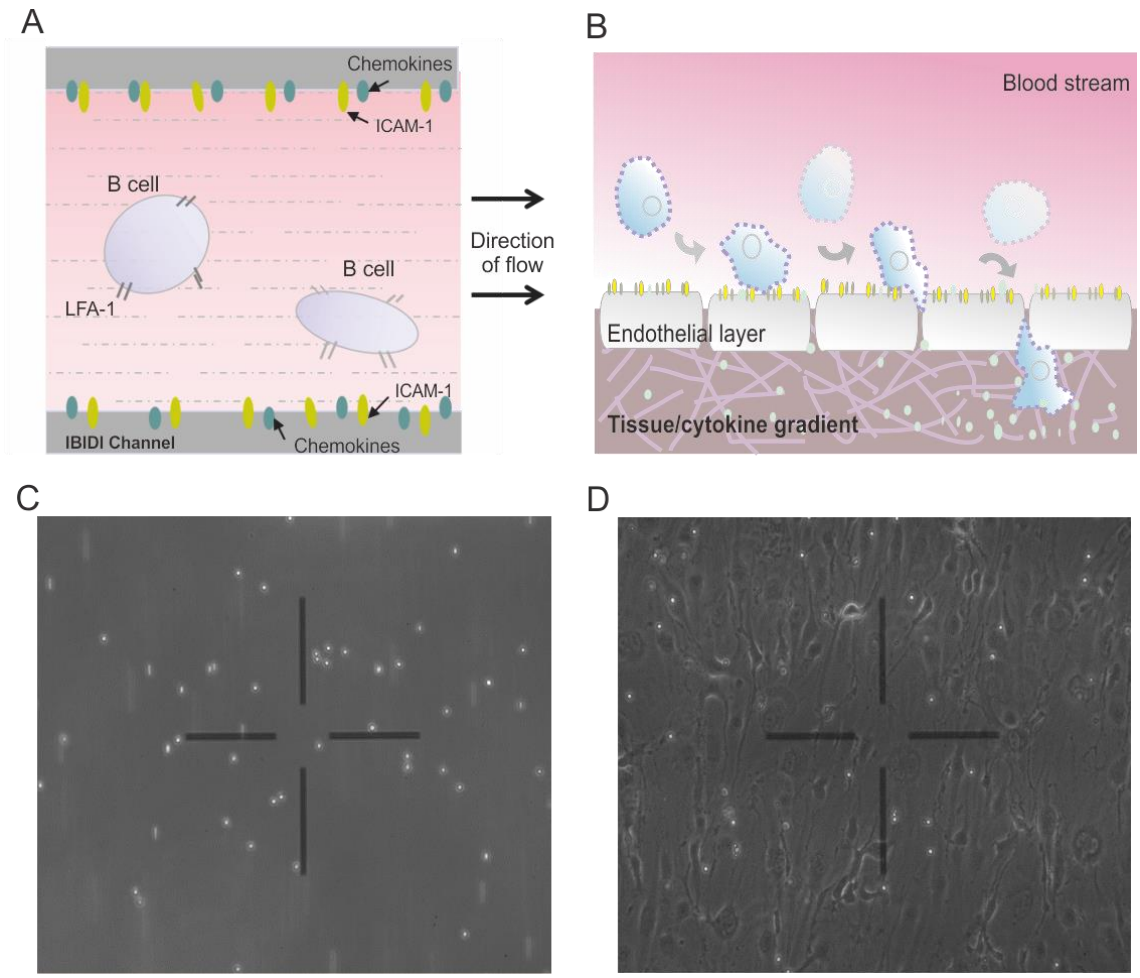


Figure 11. Graphic representation of shear flow adhesion assays and images of field of view seen during assays.

(A) Graphic representation of the shear flow adhesion assay set-up used to mimic (B) the adhesion process of leukocytes under shear flow conditions. (C) Example of a still image of the "field of view" during a shear flow experiment using murine B cells flowing through IBIDI channels coated with 6 µg/ml ICAM-1.(D)Example of a still image of the "field of view"of murine B cells flowing through IBIDI channels with bEND.3 cells grown within the channels.

Adhered cells were defined as cells that were stationary in the field of view instead of "rolling" across the surface, and with a defined cell boundary. Videos were recorded using Hamamatsu Digital CMOS camera ORCA-Flash2.8 (C11440-10C), HCImage Live Software and videos were analysed using Imaris software (Bitplane AG). Videos

of flow experiments were analysed at 1 minute or 2 minute intervals (depending on cell numbers in the frames) with 100 frames used for analysis recorded at 2725 frames per minute.

2.2.7. 2D migration assays on ICAM-1 ligands

1 μg/ml, 2 μg/ml or 6 μg/ml ICAM-1 were added into 8-well μ-slides (Ibidi) and left overnight at 4 °C to allow the immobilization of ligands onto the surfaces. Effector cytotoxic T lymphocytes were passaged the day before the experiment. After washing the wells with PBS, 70,000 cells were added to each well in a volume of 400 μl adhesion assay medium, and left to adhere for 30 minutes at 37°C. Non-adhered cells were removed by gentle pipetting, and 400μl fresh binding medium (supplemented with IL-2 and desired inhibitors) was added from the side of the wells right before the recording started. Time lapse microscopy was performed on a Nikon Eclipse Ti microscope with perfect focus and multi-point site visiting stage, using a CFI Plan Fluor ELWD 40x phase objective and a Photometrics Cascade II 1024 black illuminated EMCCD camera. Images were recorded for at least 90 minutes at 90 frames per hour. Videos were viewed on NIS-Elements Viewer and subsequently analysed using Imaris software (Bitplane AG). For analysis of cell crawling without shear stress in 2D, time frame at “Frame 100” to “Frame 200” since start of recording was analysed, approximately at the 1 hour time point which was also 30 minutes after allowing the cells to settle on immobilizd ICAM-1. Imaris Software settings were limited to analyse particles (cells) bigger than 10 μm.

2.2.8. Stimulating lymphocytes for western blot analysis and Rap1 assays

For western blot analysis to detect changes in cell signalling pathways, cells that had been pre-treated with inhibitors were stimulated using 200nM phobol ester (PDBu), 10μg/mL of stimulatory anti-BCR antibodies or 10μg/ml anti-CD3 anti-CD3 in cell adhesion assay. The cell culture flask or plate was tilted slightly, stimulants were added at base of the flask and the flask was given a gentle swirl to mix. The cells were returned to the incubator at 37 °C with 5 % CO_2 for 30 minutes. For active Rap1 detection assays, the cells were only kept at 37°C with 5 % CO_2 for 15 minutes.

To stop the cell stimulation, cells were transferred to 15ml centrifuge tubes and washed twice with DPBS by centrifuging at 300g for 5 minutes using Hereaus Multifuge 3SR at 4 °C. The cells were finally pelleted by centrifuging at max speed of 12000g for 30s at 4 °C using Eppendorf Microcentrifuge 5415R/5415D and as much D-PBS as possible was removed, after which the cells were lysed as described below.

2.2.9. Cell lysis and western blot detection

Cell Lysis

$50x10^6$ cells (B cells, splenocytes and effector cytotoxic T lymphocytes) were lysed in 1ml high salt lysis buffer consisting of 350 mM sodium chloride, 50 mM Tris pH 7.4, 50 mM sodium fluoride, 10 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF, 40 μM β-glycerophosphate and one Complete Protease Inhibitor Tablet per 10 ml of lysis buffer. The cell pellets were mixed with lysis buffer by repeated

pipetting and then left on ice for 30 minutes for lysis to occur, with agitation every 10 minutes for mixing. Bone marrow-derived macrophages and dendritic cells were lysed at $20x10^6$ cells/ml. The suspension was then centrifuged at max speed of 12000g for 15 minutes at 4 °C using an Eppendorf Microcentrifuge 5415R/5415D to pellet the debris. The supernatant e.g. cell lysate was then prepared for SDS-PAGE electrophoresis and frozen at -20 °C until.

To investigate signalling pathways in bone marrow-derived macrophages after phagocytosis challenge the cells were lysed at $2x10^6$ cells/ml in low salt lysis buffer, supplemented with 1mM sodium vanadate, 25 µl of NuPAGE LDS Sample Buffer and 10 µl NuPAGE Reducing Agent which was added to every 65 µl of lysis buffer. This lysis solution was added directly onto bone marrow-derived macrophages on 6-well plates and the plates were incubated on ice for 5 minutes. Cells were sheared using 25 G needles and 2 ml syringes (BD Falcon and Terumo) during this time. Samples were then heated at 100 °C for 5 minutes, then kept frozen at -20 °C until required for SDS-PAGE electrophoresis.

SDS-PAGE Electrophoresis

Protein samples were prepared by adding 25 µl of NuPAGE LDS Sample Buffer and 10 µl NuPAGE Reducing Agent to every 65 µl of lysate, and then heated at 70 °C for 10 minutes. 20 µl of prepared protein samples were loaded onto NuPAGE Novex 4-12 % 1.0 mm, 10 well (NP0321Box) gels. 10 µl of Pre-stained SeeBlue Plus 2 was used as protein standard. NuPAGE MOPS or MES Running Buffer was used for electrophoresis, depending on the size of proteins to be visualised. MOPS buffer was

used for general separation of proteins while MES buffer was used to allow better separation of smaller proteins. In addition, 500 ml of NuPAGE Antioxidant was added to the middle chamber and mixed with a serological pipette to maintain protein samples in the reduced state. SDS-PAGE Electrophoresis was performed using the XCell SureLock Mini-Cell from Invitrogen. The running conditions were either 150 V constant, 50 minutes for the NuPAGE MES Buffer system or 1 hour 30 minutes for NuPAGE MOPS Buffer system, respectively.

Western Blotting

For Western Blotting, the SDS-PAGE gel was assembled into XCell II SureLock Blot Module as per the manufacturer's instructions. Protran BA85 nitrocellulose membrane was pre-soaked in NuPAGE Transfer Buffer (with 1 ml of NuPAGE Antioxidant added to a litre of transfer buffer) before assembly of the sandwich and this buffer was also used in the transfer process. Sponges for the Blot module and thick Whatman filter papers were also pre-soaked in transfer buffer and as much air as possible was removed from the sponges and filter paper using a roller. To assemble the sandwich for 1 gel/blot, 3 sponge pieces were placed at the bottom of the blot module, followed by a piece of thick Whatman filter paper, then the pre-cast protein gel, the nitrocellulose membrane, another piece of Whatman filter paper and then 2-3 sponge pieces. To assemble the sandwich for 2 gels/blots, 2 sponge pieces were placed at the bottom of the blot module, followed by a piece of thick Whatman filter paper, then the pre-cast gel, the nitrocellulose membrane, another piece of Whatman filter paper and 1 sponge piece before another layer of filter paper/gel/membrane/filter paper and then 2 sponge pieces.

The transfer was then carried out at 35 V for 1 hour 15 minutes for detection of proteins less than 100 kDa. After rinsing the blot with deionised water, Ponceau S Stain (Sigma P7170) was used to check for transfer efficiency and the membrane was then rinsed a few times with deionised water to remove the stain. The blot was then trimmed and blocked with 5 % milk in PBST (phosphate buffered saline with 0.05 %Tween) for 30 minutes at room temperature with agitation. The membrane was then incubated over night with primary antibody solution in 3 %-5 % milk or BSA in PBST. The membrane was then rinsed with PBST twice to remove the primary antibodies and then washed with PBST for 25 minutes with 3 buffer changes. The membrane was then probed with secondary antibodies conjugated to horseradish peroxidase (HRP) in 3 %milk in PBST for 45 minutes. The membrane was then rinsed again with PBST twice to remove the secondary antibodies and then washed with PBST 1 hour 15 minutes with buffer changes every 15 minutes. The Amersham ECL Prime Western Blotting Detection Reagent was used for detection. A 1:1 mixture of Solution A (luminol solution) and Solution B (peroxide solution) was made up and approximately 500-750 µl of this mixture was pipetted onto a small tray and the membrane (right-side-down) was placed over this and left for 1 minute for the ECL solution to react with the conjugated HRP. The membrane soaked with ECL solution was wrapped in overhead projector film or cling film and then exposed to X-ray film (Fujifilm) in a film cassette for a suitable length of time and fixed and developed in a Kodak Imaging developer.

Stripping of blots

To reuse a blot for detection of other proteins, the bound antibodies were stripped off the blots by incubating in Stripping Buffer (0.2M Glycine or 15g, 3.5 mM SDS or 1g and 1 %Tween20 10ml per 1L of buffer, pH2.2) at 37°C for 20 minutes with a buffer change in between. The blots were then washed with PBS (2x10 minutes), followed by PBS-Tween for 2x5 minutes, before blocking with 5 %milk in PBS-Tween and subsequent reprobing with primary antibodies as described above.

2.2.10. Active Rap1 pull-down assay

Rap1 activity was determined using a Rap1 pull-down assay kit to isolate activated (GTP-loaded) Rap1 molecules (GST-RalGDS-RBD pull-down assay, Thermo Scientific). Prior to the pull-down assay, A20 cells or B cells were suspended at a concentration of 2×10^7 in 3ml of adhesion medium (RPMI 1640 supplemented with 40 mM Hepes/0.1 % BSA) per sample and rested for 15 minutes at 37 °C. Cells were then treated with inhibitors or left untreated for 30-60 minutes in T25cm^2 flasks as described earlier. The cells were then transferred into 15 ml tubes and stimulated with 200 nM PdBu or with 10 μg/mL of stimulatory anti-BCR antibodies for 5 minutes at 37 °C. To stop the cell activation, 10ml of ice cold PBS was added into the tubes. The cells were then placed on ice and washed with PBS twice by centrifuging at 300 g for 5 minutes using Hereaus Multifuge 3SR at 4 °C to remove the buffer.

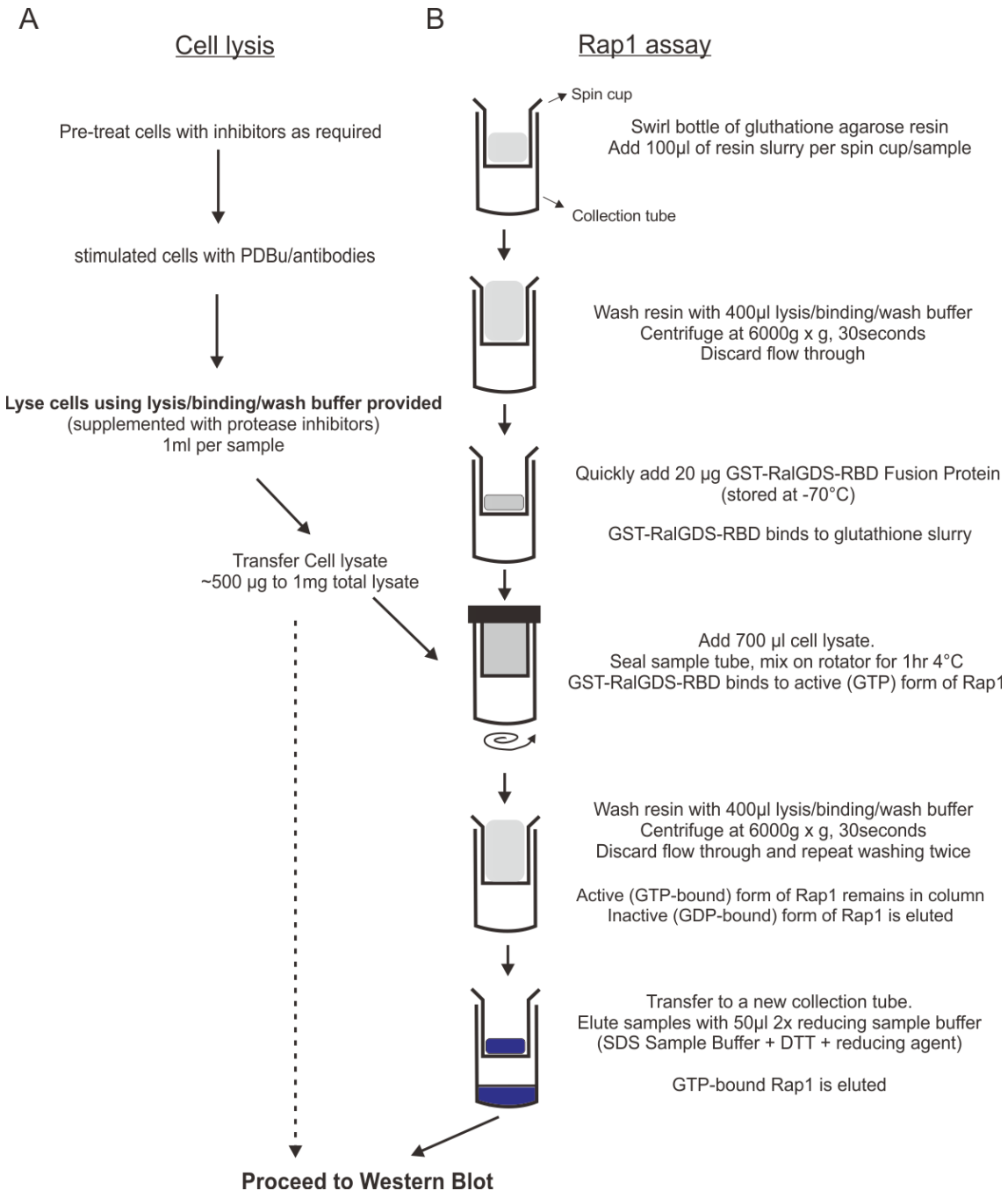


Figure 12. Graphic summary of active Rap1 pull-down assay

(A*) Cells were pre-treated with required inhibitors, stimulated, then lysed for Active Rap1 pull-down assay. Lysate were loaded into spin-cups loaded with gluthathione beads used in Rap1 pull-down assays. (B) Graphic summary of how active Rap1 pull-down assay was carried out using the kit from Thermo Scientific.*

Cell lysates were prepared and used in the pull-down assay according to the Manufacturer's instructions. One Complete Protease Inhibitor Tablet was added per 10 ml of lysis/binding/washing buffer provided in the kit (25 mM Tris•HCl, pH 7.2, 150 mM NaCl, 5 mM MgCl2, 1 % NP-40 and 5 % glycerol). Cell pellets were mixed with 1 ml of lysis buffer per sample by repeated pipetting. The lysate was transferred to a 1.5 ml microfuge tube and left on ice for 5 minutes for lysis to occur. The suspension was then centrifuged at max speed of 16000 g for 15 minutes at 4°C using Eppendorf Microcentrifuge 5415R/5415D to pellet out the debris. The supernatant of cell lysate was then transferred to a new microfuge tube. 65ul of total cell lysate was removed and transferred to another microfuge tube to be prepared for SDS-PAGE electrophoresis as described earlier (6.5 % of input lysates for the assay). The remaining cell lysate was transferred into another microfuge tube on ice while preparing the spin columns for the pull-down assay.

A spin cup was placed into a collection tube for each sample. The bottle of Glutathione Agarose Resin was swirled to thoroughly resuspend the agarose beads. 100 µL of the 50 % resin slurry was added to the spin cup with a collection tube. The tubes were centrifuged at 6000 g for 30 seconds. The flow-through was discarded and 400 µL of Lysis/Binding/Wash Buffer was added to each tube with resin. The tubes were inverted gently several times to mix. The tubes were again centrifuged at 6000 × g for 30 seconds and the flow-through was discarded.

GST (Glutathione S-transferase)-tagged fusion protein of Rap1-binding doman (RBD) from human RalGDS, or “GST-RalGDS-RBD” was thawed on ice immediately prior to the assay to make 20µg aliquots. RalGDS is also known as Ral guanine nucleotide dissociation stimulator/Ral guanine nucleotide exchange factor (RalGEF) which binds

to active, GTP-bound Rap1, but not to the GDP-bound, inactive form of the protein. The remaining aliquots for later use were stored at -70 °C. 20 µg of GST-RalGDS-RBD was added to the spin cup containing the glutathione resin, allowing the GST-fusion protein to bind to the glutathione resin. 700 µL of the cell lysate was transferred to each of the spin cups. GST-RalGDS-RBD bound to the glutathione agarose resin now binds to active Rap1, retaining active Rap1 in the spin column, while inactive (GDP) Rap-1 does not bind.

The lids were closed and the caps sealed with laboratory film before mixing the samples using a vortex. The tubes were incubated at 4 °C for 1 hour with gentle rocking. The tubes were centrifuged at 6000 × g for 30 seconds and the spin cup with resin was transferred into a new collection tube. 400µL of Lysis/Binding/Wash Buffer was added to each tube and was inverted gently three times to mix. The tubes were then again centrifuged at 6000 × g for 30 seconds and the flow-through was discarded. The washing step was repeated twice and the spin cup with resin was then transferred to another new collection tube.

Protein sample buffer was made up using LDS sample buffer, reducing agent and water in the ratio of 5:1:4 (Nupage) and 50 µl of this reducing sample buffer was added to the each spin cup containing resin. The samples were mixed using a vortex and incubated at room temperature for 2 minutes. The tubes were centrifuged at 6000 g for 2 minutes and the spin cup containing the resin was discarded. The protein samples eluted in the collection tube contained the activated (GTP loaded) Rap1. The samples were heated for 5 minutes at 95-100 °C. Total cell lysate were also prepared for SDS-PAGE gel electrophoresis. Samples were then stored in -20 °C until use.

2.2.11. Flow Cytometry

1-2 million cells per sample (including samples of unlabelled cells, cells to be labelled for individual colour compensation for multicolour flow cytometry, and actual samples for analysis) was transferred into 5ml flow cytometry tubes (352052 BD Falcon). Cells were washed twice with PBS by centrifuging at 300 g for 5 minutes using Hereaus Multifuge 3SR at 4 °C, resuspended, and mixed using a vortex after PBS addition.

The labelled antibodies were diluted according to manufacturer's recommendations and then titrated by Vicky Morrison to determine the suitable concentration for use in flow cytometry experiments. Antibodies were then used at 1:100 or 1:200 dilution in PBS (50µl per sample). Compensation controls were made up separately (consisting of 100 µl of stain solution). For bone marrow-derived macrophage and dendritic cell samples, the stain solution contained 2 mM EDTA. The stain solution was added into sample and compensation tubes. 50 µl FC block solution was also added to bone marrow-derived macrophage and dendritic cell samples to block Fc-receptors on their surface that may otherwise bind the labelled antibodies unspecifically. The cells were incubated for 20 minutes at 4 °C. The stained cells were washed with once with PBS and resuspended in 200-400 µl of PBS.

Data were acquired on an LSR Fortessa flow cytometer (Becton Dickinson), collecting 50,000-100,000 events per sample where possible and analyzed using FlowJo software (Treestar). Live cells were gated according to their forward scatter and side scatter, excluding debris. The data was then presented in a histogram where cell counts were plotted against Live/Dead stain AmCyan kit L34957 or DAPI and the "Live" cell

population was gated. This gated population was then further analysed for fluorescence, as described in the text.

2.2.12. COS-1 transfection

Square glass cover slips was added to 6 well-plates, and 2 ml of DMEM medium (with 1 0%FBS, 1 % pen/strep solution) was added to each well, ensuring that no air bubbles were trapped below the cover slips. 80 % confluent COS-1 cells in 25 cm2 flasks were detached using 1.5 ml of Trypsin EDTA for 20-30 minutes, then resuspended with another 4.5 ml of DMEM, and 150ul of the cell suspension was then added to each well. Cells were left to grow overnight before transfection.

Plasmid DNA constructs were obtained from Matthew MacPherson. Briefly, the coding sequence of CD11b and CD18 were subcloned into the multiple cloning site (MCS) of pcDNA3.1(+) and pcDNA 3.1/Hygro(+) from Life Technologies respectively. pcDNA3.1-CD11bH77 and pcDNA 3.1-CD18TTT/AAA were produced using site-directed mutagenesis. Plasmids were purified using Qiagen Plasmid Maxiprep according to the manufacturer's protocol. The constructs were checked using sequencing analysis. DNA sequencing analysis was carried out by the Genetics Core Services Unit, School of Medicine, University of Dundee, UK.

Transient transfections were carried out using FuGENE® 6 Transfection Reagent. Plasmid DNA was mixed with FuGENE® 6 Transfection Reagent (Roche) for transfection according to manufacturer's instructions. COS-1 cells were transfected with CD11bWT/CD18-TTT or CD11bWT/CD18-AAA, or CD18WT/CD11b-R77 or

CD18WT/CD11b-H77 constructs. All plasmid DNA was diluted to 0.5 µg/µl concentration. Approximately 3 µg of plasmid DNA was used for integrin subunit transfections and the amounts were adjusted slightly after flow cytometric analysis to ensure for equal surface expression of both integrin subunits.

6 µl of FuGENE® 6 was added into 100 µl Opti-MEM (31985047) per transfection reaction (without touching the sides of the tube) and incubated for 5 minutes. Plasmids were added into the mixture and incubated at room temperature for 30 minutes. 100ul of the mixture was then added slowly in a drop-wise manner into each well. The plates were swirled gently to mix and the cells were incubated at 37 °C with 5 % CO_2 for 48 hours before being used in phagocytosis assays. The transfection efficiency was assessed using flow cytometry with integrin-specific antibodies. The LFA-1 integrins were expressed on the surface of 35-55 % of the cells, while Mac-1 integrins were expressed on the surface of about 30-40 % of the transfected COS-cells. (Results, Chapter 5.)

2.2.13. Phagocytosis assay

Mac-1-mediated phagocytosis assays were performed as described by Chow et al., 2004. 300 µl of 10% Sheep Blood Red blood cells (RBC) in Alsever's solution (SB069 from TCS Biociences), were transferred into 1.5ml microcentrifuge tubes. Cells were washed with ice cold PBS (without calcium or magnesium ions) once and then twice with ice cold GVB Buffer (which prevents cell aggregation and spontaneous cell lysis), and centrifuged at 1500g for 30 seconds at 4 °C using Eppendorf Microcentrifuge 5415R/5415D. The cells were resuspended in 250 ul of GVB Buffer and 2 ul of rabbit

anti-sheep RBC IgM (CL9000-M, rabbit polyclonal IgM from Cedarlane) stock, which is a strong activator of the complement cascade. The red blood cells were incubated at 37 °C 1 hour on an end-over-end rotator, during which time the IgM coats the RBCs. The sheep RBCs were then washed 3 times with ice cold GVB, resuspended in 225ul cold GVB with added 25 µl (10 % v/v final concentration) of C5-deficient human serum (Calbiochem) and incubated at 37 °C 20 minutes on an end-over-end rotator to opsonise sRBCs. The C5-deficient serum was used to prevent cell lysis due to formation of the membrane attack complex in the complement cascade. The cells were washed with ice cold GVB, and then twice with ice cold PBS.

Meanwhile, transiently transfected COS-1 cells grown on cover slips as described earlier were stimulated by replacing the cell culture medium with 2 ml DMEM containing 100 nM PMA (phorbol 12-myristate 13-acetate) for 20 minutes at 37 °C to activate the Mac-1 integrin on the cells. Although COS-1 cells are not macrophages, these fibroblast cells can be easily manipulated by transient transfection and the cell cortex regions could be observed clearly when these cells adopt a well spread-out morphology. 500 µl of complement-coated sRBCs (2x109/ml) in DMEM (with 100 nM PMA) was added to each sample of transfected COS-1 cells in 6 well-plates. Plates were then incubated in the 37 °C incubator for 20 minutes, allowing phagocytosis of C3bi opsonized sheep RBCs to occur. To stop the phagocytosis process, the medium was removed from the 6-well plates and 2 ml of water was added to lyse non-internalised sRBC by osmotic lysis for 1 minute. The cells were washed twice with PBS and fixed with 1ml pre-chilled methanol per well for 20 minutes.

For bone marrow-derived macrophages, phagocytosis assays were performed as above, with the following modifications. Square glass cover slips were added to 6 well-plates,

after which 2 ml of DMEM medium (with 10 % FBS, 1 % pen/strep solution) was added to each well, ensuring that no air bubbles were trapped below the cover slips. Bone marrow-derived macrophages (on day 6 of culture) were harvested and reseeded into these 6-well plates at 1.5 million cells per well in 2 ml medium. Cells were left to grow overnight before the phagocytosis assay was carried out. The IgM used to coat RBCs in these assays was Mouse anti-sheep IgM MGM00 (Life Techologies) instead of rabbit polyclonal IgM mentioned earlier but were also treated with the same C5-deficient serum for to opsonise sRBCs.

Before the addition of complement-coated RBCs, macrophages were serum-starved by replacing the cell culture medium with 2 ml of DMEM and incubating the cells in the incubator for 1hr. Macrophages were stimulated with 100nM PMA in DMEM for 30 minutes. Thereafter, 500 µl of complement-coated sRBCs (2x10^9/ml) was added to each sample of macrophages in 6 well-plates, and the phagocytosis was allowed to occur for 15 minutes.

2.2.14. Dextran Uptake assay

Bone marrow-derived macrophages were harvested on day 7 of culture and 1 million cells per sample in pre-warmed 200 µl of RPMI medium with 10 % FBS, 1 % pen/strep solution and 10 ng/ml M-CSF were transferred into 5ml FACS tubes. The cells were stimulated with 100 nM PMA for 20 minutes in 37 °C with 5 % CO_2. Dextran Fluorescein 70000 MW labelled with FITC (Life technologies D-1822) was reconstituted at 10 mg/ml in PBS and wrapped in foil to protect from light. This FITC-Dextran was diluted 1:5 in the same RPMI medium used for suspending the cells to get

2 mg/ml of FITC-Dextran for the assay. 200 µl of FITC-Dextran was added into the sample tubes and these were incubated for 10 minutes at 37 °C. Control tubes were placed on ice after the addition of FITC-Dextran. Cells were washed thrice with ice cold cell medium to remove excess Dextran. The cells were resuspended in PBS and analysed by flow cytometry. The mean fluorescence intensity of the samples was analysed using FlowJo to compare Dextran uptake.

2.2.15. Immunofluorescence and confocal microscopy

At the end of the phagocytosis assays, 2ml of ice-cold methanol was immediately added into the wells in the 6-well plate that contained the cover slips to fix the cells. The plates were placed on ice for 20 minutes. The cells were then rinsed with 2 ml PBS, taking care not to dislodge the cells grown on the cover-slips. Unspecific binding of antibodies was blocked using 1 ml of PBS supplemented with 2% goat serum and 0.1 % triton x-100 for 10 minutes at room temperature. Transfected cells that were subjected to phagocytosis assays were labelled with FITC-conjugated C3c antibody (Dako) which binds to iC3b-opsonized RBCs or with Anti-CD18 antibody [C71/16] (ab19580) (which is then labelled with secondary antibodies (Anti-Rat IgG–FITC antibody from Sigma).

Primary antibodies Anti-CD18 were made up in blocking solution at 1:100 (in PBS supplemented with 2 % goat serum and 0.1 % triton x-100) and 50 µl of this solution was pipetted gently to form a droplet on a piece of parafilm laid on a flat surface. Cover slips were removed using fine tweezers and gently placed on top of the drop of primary antibody on the parafilm, ensuring no air bubble was trapped in between. The cover slips were left for 45 minutes at room temperature. The cover slips were picked up

from the parafilm using tweezers on one hand and a P200 tip as a support held by the other hand. To wash the cover slip, the cover slip was dipped into 300 ml of PBS in a beaker with gentle agitation, the washing procedure was repeated in another beaker and the coverslip was dried by resting the edge of the cover slip against paper towels. Application of the secondary antibody, Anti-Rat IgG–FITC antibody at 1:100 was performed the same way as for the primary antibodies. The cover slips were washed in PBS and dried by wicking on paper towels. FITC-conjugated C3c antibody was also made up in PBS supplemented with 2 % goat serum and 0.1 % triton x-100 in a 1:50 dilution for complement staining of RBCs but the incubation time was 30 minutes at room temperature. The cover slips were washed in PBS in beakers and dried as before. 20 µl of Fluoroshield^{TM} with DAPI (F6057, Sigma) was placed on glass microscopic slides (FisherScientific). The cover slips were placed slowly onto the drop of Fluoroshield^{TM} using tweezers with care taken to prevent any trapped air bubbles from forming. The cover slip was sealed onto the glass slides using Glycerol Gelatin aqueous slide mounting medium (GG1, Sigma) and left to dry overnight.

Microscopic slides were observed and images recorded using a Leica TCS SP5 confocal microscope. Images were processed using LAS AF software. After counting the total cells present in each frame and taking into account that only a certain percentage of cells were successfully transfected, the phagocytosis index was calculated by dividing total number of red blood cells by number of phagocytic cells (over 3-5 fields of view per sample) and multiplying it by 100, expressing it as the number of red blood cells ingested by 100 phagocytic cells.

2.2.16. Statistical analysis

The Student's t-test (Excel) 2-way distribution, paired variance and two-way ANOVA (Graphpad Prism) tests were used to calculate p-values. Results were considered significant if the p value was less than 0.05.

3. Regulation of integrin-mediated adhesion in primary murine B cells

3.1. Introduction

3.1.1. B cell adhesion and migration

Integrin-mediated cell adhesion is important for B cell development in the bone marrow, after they leave the bone marrow for B lymphocyte recirculation, migration into secondary lymphoid organs such as lymph nodes and spleen via HEV, activation of B cells and effector functions in the immune system (Mauri and Bosma, 2012, Tobón et al., 2013).

3.1.2. The B cell immunological synapse

The LFA-1 integrin is important in the formation of the immunological synapse between helper T cells and B cells during activation. Stimulation of the B cell receptor leads to the activation of Btk, the Src family kinase Syk, Lyn and Vav, which activate 3 other signalling pathways via Btk, PI3K and PLCγ2 (Arana et al,. 2008, Woyach et al., 2012). These pathways then activate Rap1, which regulates integrin-mediated cell adhesion (Arana et al., 2008). It was found that mutation of PI3K p110δ did not affect T cell integrin-mediated cell adhesion although T cell activation was impaired (Okkenhaug et al., 2002). B cells lacking PI3K p110δ had impaired Btk and PLCγ2 signalling, making them prone to apoptosis and affecting cell proliferation (Clayton et al., 2002).

LFA-1 and VLA-4 integrins have also been found to be important in mediating antigen presentation between B cell and antigen-presenting cells or helper T cells, so that only small amounts of antigen is required to effectively stimulate B cells at the immunological synapse (2006 Arana et al., 2008). The antigens are gathered in the cSMAC (central supramolecular activation cluster) in the presence of VLA-4, while a ring of LFA-1 integrins is formed in the pSMAC (peripheral supramolecular activation cluster) to promote cell-cell contact between the helper $CD4^+$ T cell and B cell. (Carrasco et al., 2004, Carrasco and Batista, McGinn et al., 2011).

3.1.3. B cell migration within tissues

Naïve B cells entering the lymph nodes migrate to follicles where they are exposed to follicular dendritic cells or Helper T cells. Migration to follicles is regulated by CXCR5 receptors following CXCL13 ligands while migration to T cell zones is regulated by CCR7 along CCL21/CCL19 gradients (Okada and Cyster, 2006). Cells that do not encounter antigen will re-enter the circulation via the lymphatics. The spleen filters blood and consists of a large population of B cells (Pillai and Carriapa, 2009). B cells in the marginal zone (MZ) are located outside the marginal sinus surrounding the white pulp cords of the spleen. These MZ B cells are constantly exposed to antigens from the circulation and are generally not recirculatory. These cells have increased expression of LFA-1 and VLA-4, which allows for the MZ B cells to be retained in this area (Lu and Cyster, 2002, Okada and Cyster, 2006).

3.1.4. B cell emigration from the bloodstream

Selectins and integrins are involved in the leukocyte extravasation process, as leukocytes in the blood stream first slow down and roll across the endothelial cell layer via selectins before cell arrest due to integrin-mediated cell adhesion (McDonough et al., 2004). B cells deficient in Class 1A PI3K p110δ or pretreated with PI3K inhibitor, wortmannin, show reduced homing to Peyer's patches and splenic white pulp cords in adoptive transfer experiments and reduced chemotactic responses to CXCL13 (Reif et al., 2004). Talin-1, a co-activator of integrins and a cytoskeletal adaptor, was needed for B cell emigration to lymph nodes. B cells lacking talin-1 were not able to enter the lymph nodes or return to the bone marrow as integrins were unresponsive, but chemotaxis was unaffected (Manevich-Mendelson et al., 2010). The Ras-like GTPase Rap1A, another activator of integrins was shown to be necessary for B and T cell adhesion to fibronectin and ICAM-1 but loss of Rap1A can be compensated by Rap1B and other Rap1 independent signalling pathways as homing of B cells and T cells to lymph nodes was unaffected (Duchniewicz et al., 2006).

The presence of shear flow is an important factor in cell adhesion. Low shear stress has been suggested to stabilize integrin conformation in the active state (Alon and Dustin, 2007). Cells need to overcome shear stress of up to 5 dynes/cm^2 in vivo in the blood stream during the extravasation process (Morigi et al., 1995). However, comparisons between the presence and absence of shear flow in the regulation of integrins have not been extensively performed.

3.2. Aims of this Chapter

The aim of this chapters was to investigate the role of AGC kinase family members and putative downstream effectors in regulating integrin-mediated B cell adhesion with and without the presence of shear flow, mimicking conditions in blood vessels or in tissues, respectively.

3.3. Results

3.3.1. B cells isolated from mouse spleens were of high purity

In order to study integrin-mediated adhesion in B cells, B cells were isolated from mouse spleens. Approximately 47 x10^6 B cells can be isolated from mouse spleens, (40-60 % of splenocytes) (Hsueh et al., 2002). Alternatively, non-purified, B cell-enriched splenocytes were used after lysing red blood cells and removing most monocytes and macrophages (by letting the suspension of spleen cells adhere to tissue culture plates) (Hwang et al., 2004). However, for specificity and quality of experimental results, isolation of B cells from spleens was carried out when possible. Primary B cells were isolated from spleens from 6-8week old C57BL/6 mice using Dynabeads Mouse Untouched CD43 (Untouched B cells) magnetic beads following the manufacturer's instructions. The purified cells were analysed for the expression of B cell marker, CD19, using flow cytometry to assess the purity of B cells before and after the purification process (Figure 13).

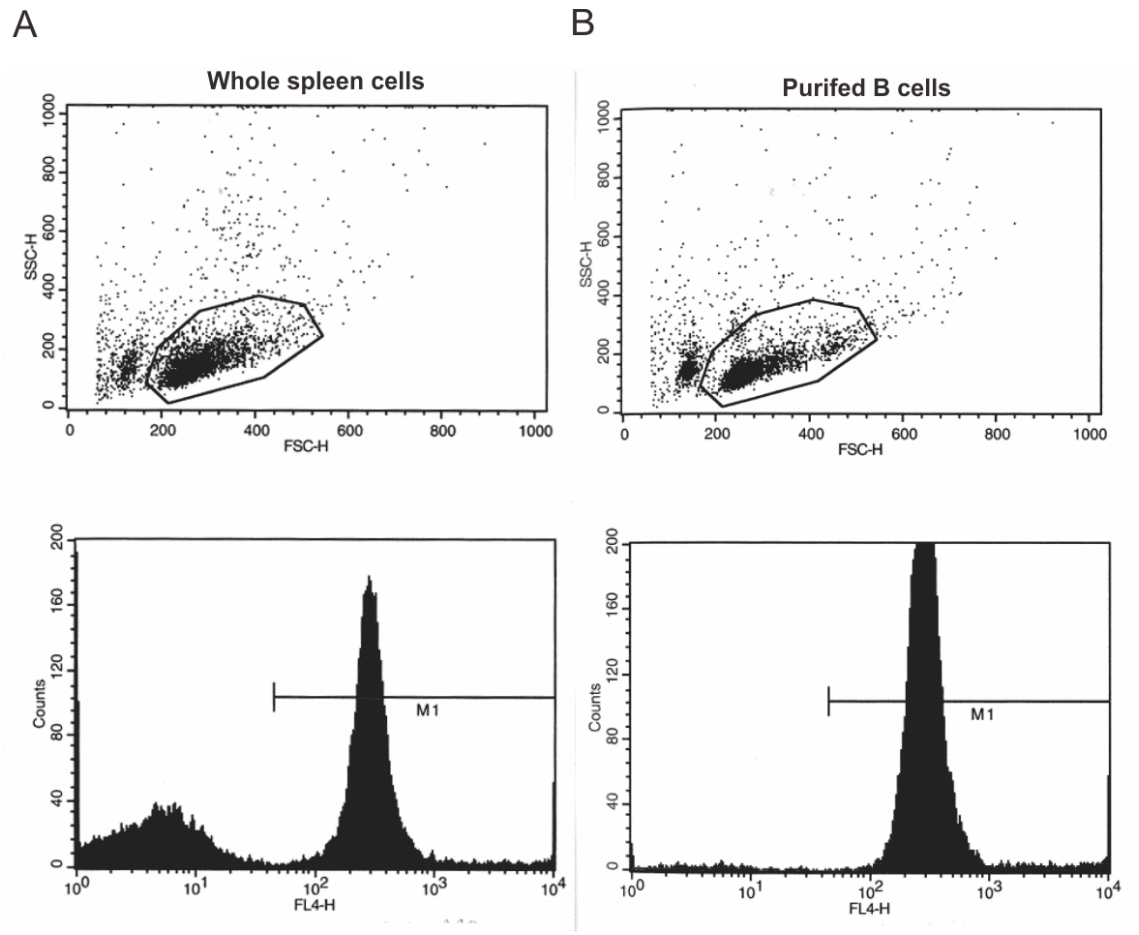


Figure 13. Flow cytometric analysis of CD19 positive primary B cells from spleens of wild-type mice before and after purification using untouched CD43 beads.

(A and B: top) Cells are shown in a dot display plot and "B cells", excluding debris, were gated accordingly. (A and B: bottom) The fluorescence (CD19 expression) of the gated cells was then displayed as histograms. (A) Represents spleen cells before purification. (B) Represents B cells after purification. Results are from 1 experiment. Similar results were obtained in 3 experiments, carried out with Mark Anderson/Sharon Matthews.

The population of spleen cells that were not yet purified using CD43 untouched B cell beads had 68.6 % B cells, while the population of cells after using CD43 untouched B cell beads had 98.09 % B cells (Figure 13). Results were consistent between purifications, and the overall purity was 97% +/- 2%. (Results not shown.) This result

demonstrated that purification of B cells using the CD43 untouched B cell beads resulted in highly pure B cells to be used in experiments.

3.3.2. LFA-1 integrin expression in B cells

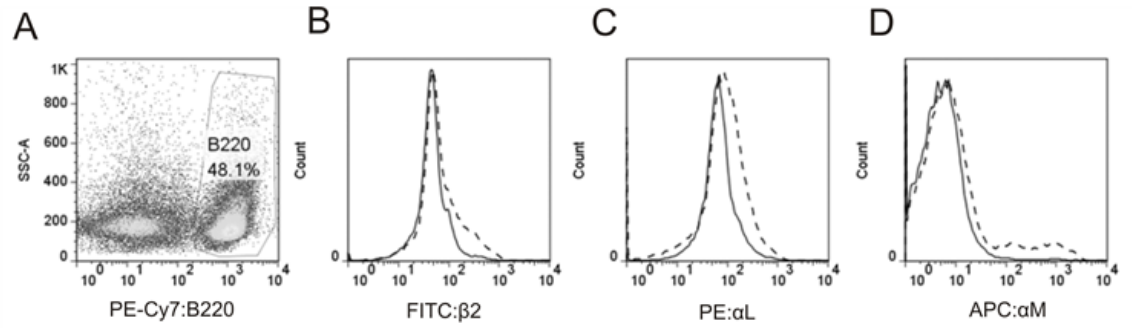


Figure 14. Integrin expression on the surface of spleen cells from wild-type mice.

(A) "Live" cells were gated from debris. Cells stained with AmCyan kit (Molecular Probes) for live/dead cells are then gated for live cells. The percentages of B220 positive B cells are shown after gating of the B220 positive B cells from the spleen cell population in scatter plot. (B-D) Histograms showing the levels of (B) β2 integrins, (C) αL integrins and (D) αM integrins, on live B220 positive B cells (solid lines) and live splenocytes (dashed lines). (Results are representative of N=2.)

The levels of integrins (β2, αL and αM integrin chains) expressed on the surface of B cells and splenocytes were then analysed by flow cytometry. B220 is another common B cell marker, similar to CD19 which was used in the previous experiment (Figure 13 amd 14). B220 is expressed mainly in B cells but a sub population of B220-positive cells has been found to have dendritic cell characteristics, while CD19 is thought to be expressed in commited B cells (Nikolic et al., 2002). Both markers are commonly recommended to identify B cells in flow cytometry.

The expression of β2 integrins on B220 positive B cells and splenocytes were very similar, while the expression of αL integrins was slightly higher in splenocytes than in B220 positive B cells (Figure 14B and 14C). Both B220 positive B cells and splenocytes expressed very low levels of αM integrins (Figure 14D).

3.3.3. Phorbol ester or B cell receptor stimulation increases cell adhesion to integrin ligands.

To investigate the regulation of primary B cell adhesion to integrin ligands, static adhesion assays were set up by coating ligands (fibronectin or ICAM-1) on 96-well plates and then allowing splenocytes to adhere to the immobilized ligand for 30 minutes. In these experiments, cells were either left unstimulated, stimulated with phorbol ester to mimic diacylglycerol and directly activate PKC, or stimulated by triggering their B cell receptor using IgM. Splenocytes from wild-type mice were used for these preliminary experiments as approximately 60 % of these cells were B cells (as shown in Figure 12), which was considered pure enough for these pilot experiments. Non-adherent cells were then gently washed off in phosphate buffered saline. Adherent cells were detected using a phosphatase substrate, p-Nitrophenyl Phosphate (PNPP), which forms a coloured product when reacted with phosphatases released from the adherent cells through lysis in sodium acetate and Triton-X-100 containing lysis buffer.

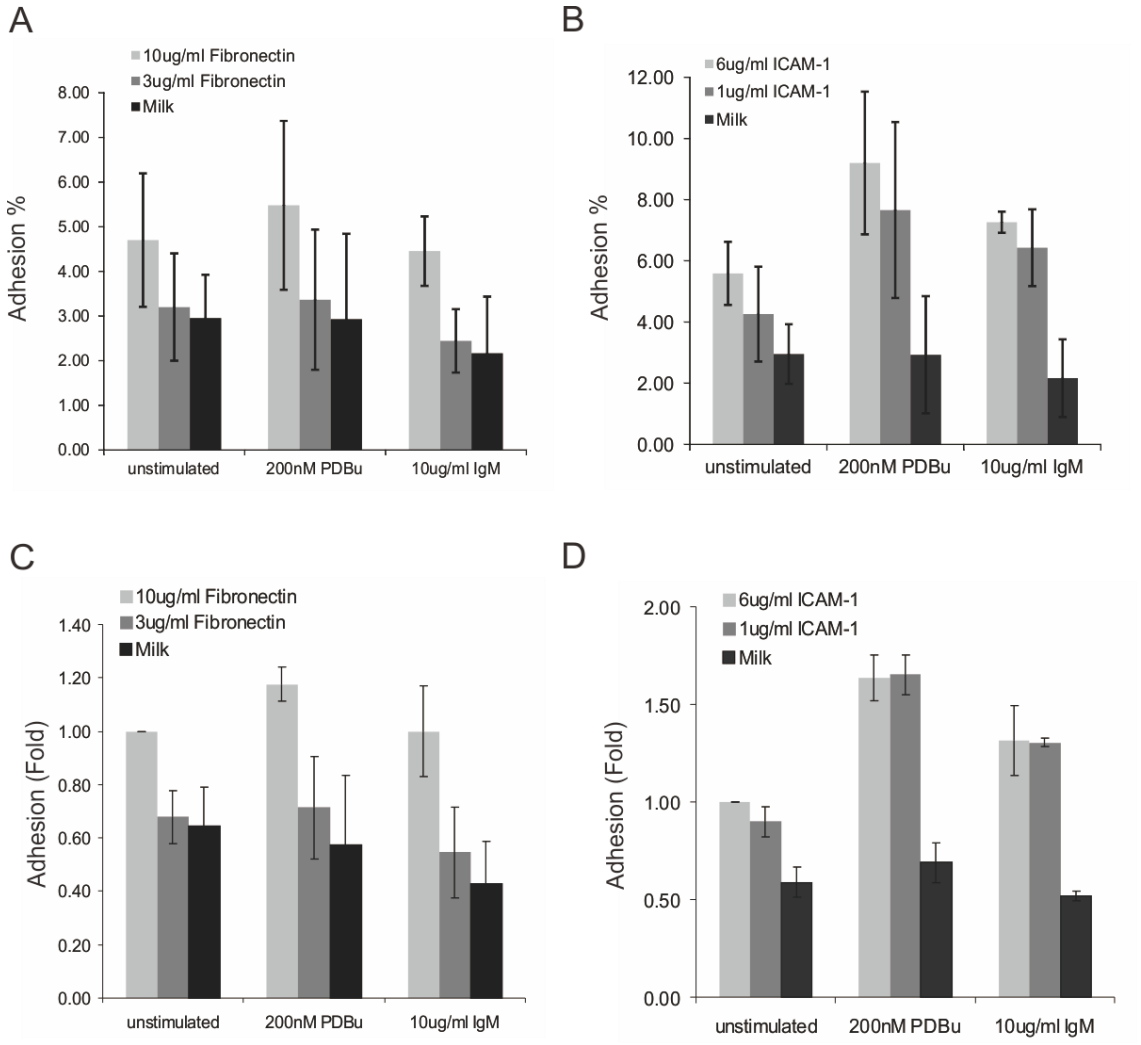


Figure 15. Assessment of murine splenocyte cell adhesion to immobilised ligands (fibronectin and ICAM-1) at various concentrations, with and without cell stimulation to stimulate integrin-mediated cell-adhesion.

(A) Adhesion of splenocytes from wild-type mice todifferent concentraions of fibronectin or to a control surface blocked using milk, without immobilized fibronectin. N=3 mice, 1 experiment. (B) Adhesion of splenocytes from wild-type mice to different concentrations of ICAM-1s or to a control protein-coated surface without ICAM-1 (milk). N=2 mice. (A-B) Results are expressed in percentage of cells adhered to immobilized ligands.(C-D) Results are expressed as "fold change" where readings from non-stimulated cells adhered to ligands is "1". Error bars represent standard deviation. Experiments were done together by Susanna Fagerholm, Vicky Morrison and Hwee San Lek.

ICAM-1 is a ligand for the LFA-1 (αLβ2) integrin while fibronectin is a ligand for the β1 integrins such as VLA-4 (Humphries et al., 2006). The optimum amount of ligands needed for this type of static adhesion assays has been demonstrated in Fagerholm et al., (2006), Matthews et al., (2012) and was also demonstrated in Figure 14. 10 µg/ml of fibronectin was needed for cell adhesion and decreasing fibronectin to 3 µg/ml significantly reduced cell adhesion to a level which is similar to the adhesion to a non-specific surface (milk-bound wells) (Figure 15A and 15C). This demonstrated that splenocytes express other integrins which bind to fibronectin in this assay. However, coating of wells with either 6µg/ml ICAM-1 or 1 µg/ml ICAM-1 resulted in similar levels of cell adhesion (Figure 15B and 15D), demonstrating that even low levels of ICAM-1 could sustain B cell adhesion.

Splenocytes that were left unstimulated had the ability to adhere to both fibronectin and ICAM-1. Stimulation of cells with phorbol ester or through the B cell receptor did not significantly increase cell adhesion to fibronectin. In contrast, stimulation of splenocytes with phorbol ester, but not BCR ligation, significantly increased adhesion of splenocytes to ICAM-1 ($p=0.009$ for 1 µg/ml ICAM-1 and $p=0.08$ for 6 µg/ml ICAM-1). Taken together, splenocytes which were stimulated by phorbol ester were more adhesive than those stimulated through the B cell receptor (Figure 14). The results also show that this static adhesion assay using fibronectin or ICAM-1 is a suitable method to assess for fibronectin binding integrin (such as β1 integrins) mediated cell adhesion or LFA-1 integrin-mediated adhesion of splenocytes or B cells.

3.3.4. Roles of AGC kinases in the regulation of integrin-mediated murine B cell static adhesion

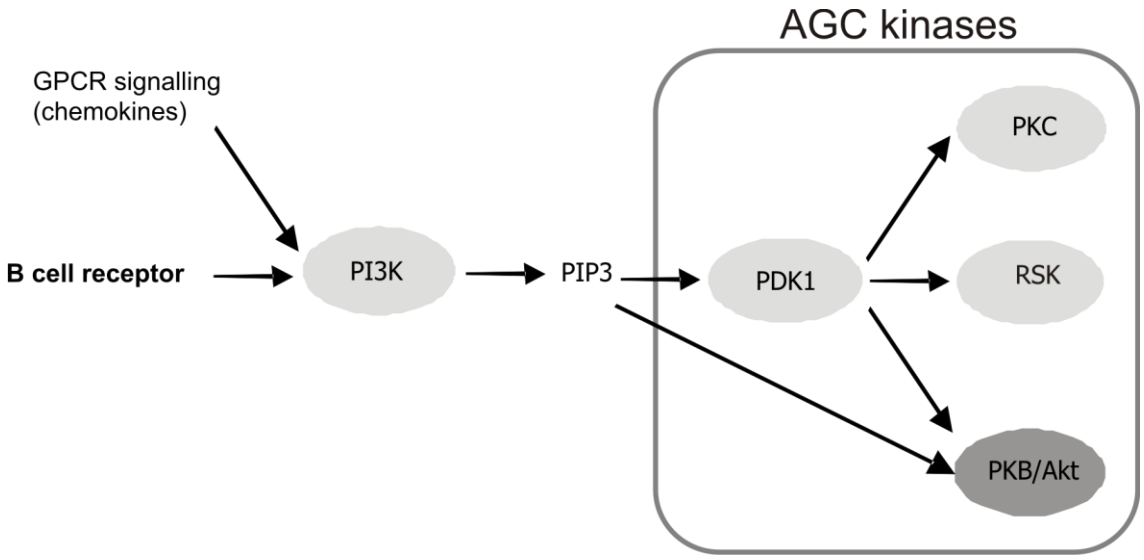


Figure 16. Schematic representation of B cell receptor signalling that activates AGC family kinases via PI3K signalling

We were interested in studying if AGC kinases play a role in regulating integrin-mediated adhesion in B cells (Figure 15). When B cells are triggered at the BCR by IgM, this leads to the activation of PI3K (Class 1A), which converts PIP_2 to PIP_3 by phosphorylation. PIP_3 production leads to the activation of PDK1, which is a central regulator of AGC kinases such as RSK and S6K (Lawlor et al., 2002). PIP_3 will be localised to the membrane to recruit and activate other kinases via Plekstrin Homology (PH)-domains, such as Akt, Btk1, PDK1 (Czech, 2000). PDK1 and PI3K signalling also regulates PKCζ (Chou et al., 1998).

Non-specificity of kinase inhibitors

In order to study if AGC kinases play a role in integrin-mediated B cell adhesion, protein kinase inhibitors targeting individual AGC kinases were used. Most inhibitors have been found to be non-specific despite manufacturers' claims. Therefore careful considerations have to be taken when deciding which kinase inhibitors to use (Davies et al., 2007, Bain et al., 2007). Cohen (2010) has also suggested essential criteria in the selection of kinase inhibitors. The inhibitor of choice had to be tested against a large panel of kinases which can be obtained from the International Centre for Kinase Profiling website (http://www.kinase-screen.mrc.ac.uk/) based in the Protein Phosphorylation and Ubiquitylation Unit at the University of Dundee. Cellular effects observed should be observed with the use of at least 2 structurally unrelated inhibitors and should be effective at the concentration similar to which phosphorylation of the kinase is inhibited. Analogues of the inhibitor that do not inhibit kinase activity in vitro should not exert the same cellular effect observed with the inhibitors. Also, the inhibitor of choice should have been confirmed and published by other researchers (Cohen, 2000). Although these criteria makes it difficult to select for high selective inhibitor that meets all of the above requirements, relatively unspecific kinase inhibitors can still be used to exclude the involvement of protein kinases in biological processes (Davies et al., 2000). Therefore, in most of the experiments in this thesis, 2 inhibitors that target each kinase were used to reduce the chance that the experimental results observed were only due to unspecific inhibition of kinase activity.

3.3.4.1. Gö6976 and Ro-31-8220 are effective PKC inhibitors in B cells

Protein kinase C isoforms are members of the AGC kinases, and have been previously implicated in integrin regulation. PKCθ, which is the major PKC isoform in T cells, is important in the regulation of integrins in T cells (Letschka et al., 2008). Therefore, we started our studies of the role of AGC kinases in B cell adhesion by investigating the role of PKC in this process. The BCR in B cells can trigger the activation of PKC independent of PDK1 (Wood et al., 2007). Phospholipase-C gamma is phosphorylated at tyrosine sites after BCR triggering, leading to cleavage of PIP2 to form membrane-bound DAG and IP3 in the cytosol. DAG and/or Ca^{2+} are needed to activate PKC, which in turn activates PKD. PDBu mimics the structure of diacylglycerol (DAG) and is used to stimulate PKC activation in cells; phorbol ester also stimulates B cell adhesion (Steinberg et al., 2008) (Figure 15 and 16).

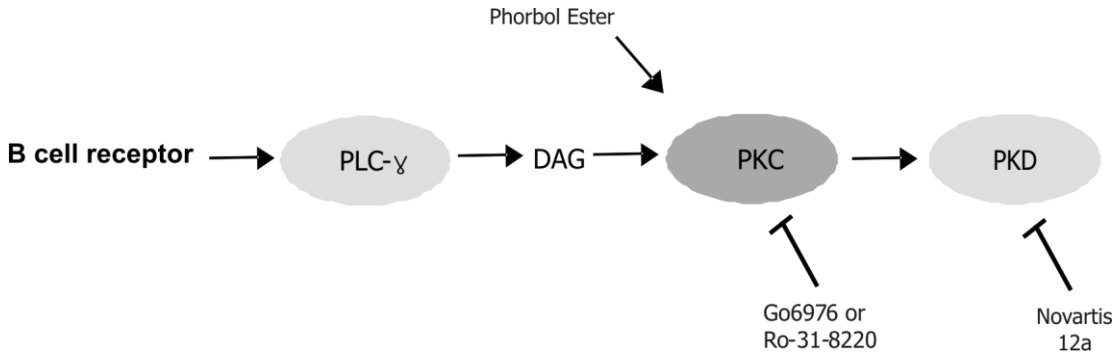


Figure 17. Schematic representation of the BCR-induced PKC/PKD pathway and relevant inhibitors used in this study.

There are at least 9 isoforms of PKC (Steinberg 2008). Gö6976 inhibits mainly classical PKCs (PKCα, PKCβ$_1$) and atypical PKCμ which is also known as PKD (Martiny-Baron et al., 1993). Ro-31-8220 inhibits all 3 classical PKCs (PKCα, PKCβ$_1$, PKCβ$_2$ and PKCγ) as well as the novel PKC isoform, PKCε (Wilkinson et al., 1993, Davies et al., 2000, Spitaler and Cantrell 2004). Research has shown that Gö6976 can also inhibit CHK1 and PHK while inhibiting PKCs. Ro-31-8220 can also inhibit GSK3, S6K, RSK and MSK among other kinases (Davies et al., 2000, Bain et al., 2007). As Davies et. al., (2000) has suggested that although kinase inhibtors are not as specific as marketed, two or more structurally unrelated inhibitors could still be used to exclude the involvement of a kinase controlling a cellular process, therefore Gö6976 and Ro-31-8220 were chosen for the initial screening for the involvement of PKC in integrin-mediated cell adhesion (Figure 17). Gö6976 is a staurosporine-related compound, with an intact aromatic ring and is an ATP-competitive inhibitor (Gschwendt, et.al, 1996). Ro-31-8220 is a bisindolemaleimide that does not have an intact aromatic ring and is also an ATP-competitive inhibitor (Powell et al., 2003) (Table 3).

In order to demonstrate that effective doses of inhibitors were used to inhibit target kinases in B cells, dose-response experiments were carried out. PKC is upstream of PKD and PKC inhibition would therefore lead to less activation and less phosphorylation of PKD in cells. Therefore, the readout for PKC inhibition in this system is the difference in levels of phosphorylated PKD (S916) detected by western blotting. In addition, we measured the difference in integrin-mediated cell adhesion using the same inhibitors.

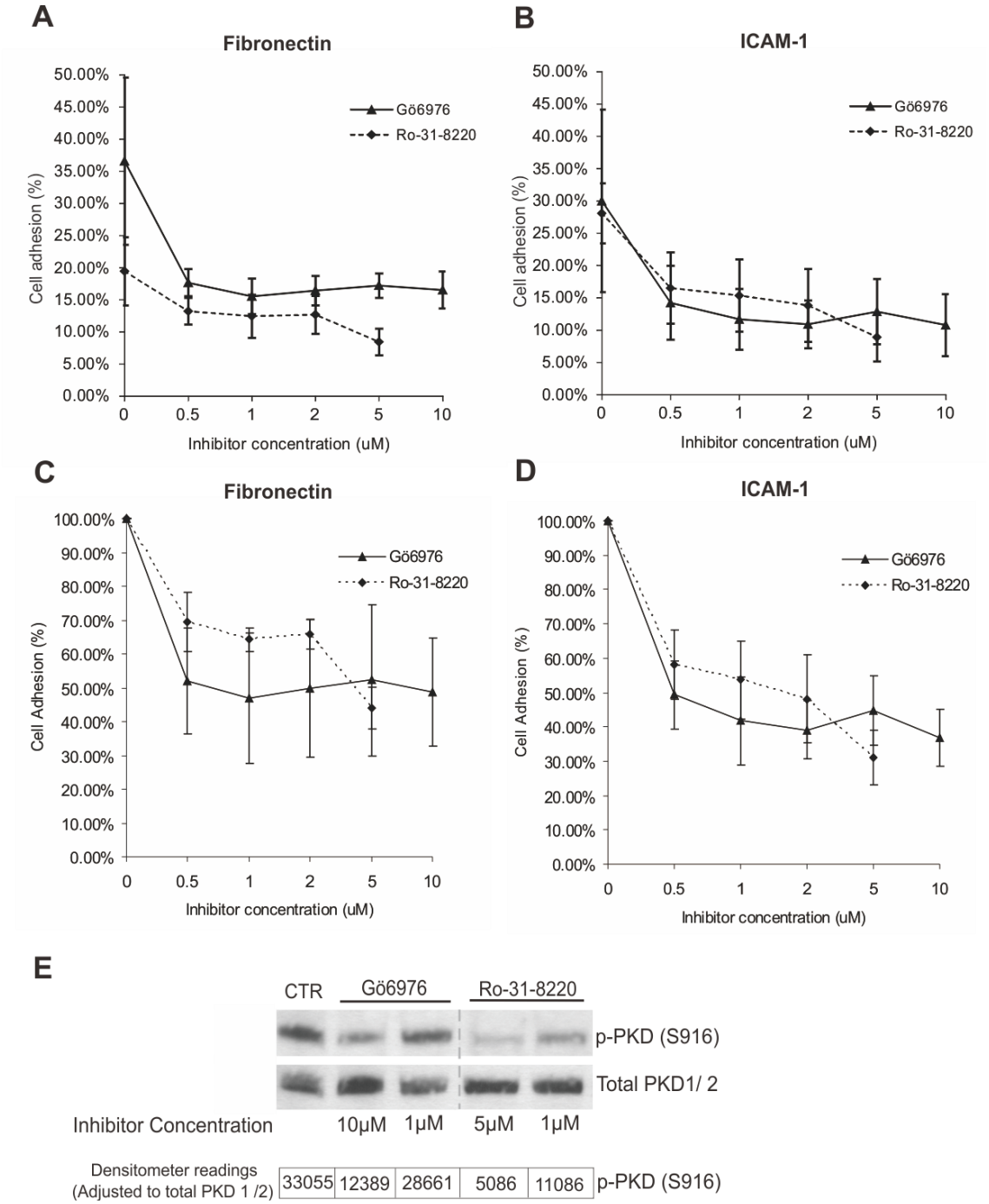


Figure 18. Effect of PKC inhibitors Gö6976 and Ro-31-8220 on PKC activity and B cell adhesion.

Dose-dependent inhibition of BCR-stimulated B cell adhesion to (A and C) fibronectin, (B and D) ICAM-1 after treatment of cells with PKC inhibitors, Gö6976 or Ro-31-8220. (A and B) Data showing percentage of adhered cells in different conditions. (C and D) Percentage decrease in cell adherence after drug treatment was plotted after calculation from data in A and

B. N=3 experiments for both treatments, where each experiment used cells pooled from 2 or 3 mice to obtain enough purified B cells. Error bars represent standard deviation. Results are expressed as percentage decrease in cell adhesion when compared to adhesion levels of untreated cells. (C) PKD activation (S916-PKD) as readout of PKC activation in B cells, after pre-treatment with PKC inhibitors at various concentrations. Cells were all stimulated through the BCR using IgM. The blots were stripped and total levels of PKD1/2 were detected and used as loading controls. Densitometer readings were carried out and readings for phospho-PKD (S916) were normalised to total PKD levels. The blots shown are are representative of N =2 experiments.

Murine B cells pre-treated with either of the PKC inhibitors showed a significant decrease in cell adhesion to fibronectin (Figure 18A and 18C) and ICAM-1 (Figure 18B and 18D) ligands and this could be observed at concentrations as low as 0.5 μM. This low concentration which was highly efficient at inhibition of integrin-mediated cell adhesion is 20x less than the recommended concentration for Gö6976, which is 10 μM. For Ro-31-8220, the recommended concentration is 5 μM and this is indeed the most effective for inhibition of integrin-mediated cell adhesion. At lower concentrations, Gö6976 was more effective in inhibition of integrin-mediated cell adhesion than Ro-31-8220 but at the recommended dose of Ro-31-822 at 5μM was more effective in inhibition of integrin-mediated cell adhesion (Figure 18A and 18B).

The levels of active, phosphorylated PKD in B cell lysates in PKC inhibitor-treated cells were lower than in untreated cells. Treatment of cells with high (recommended) concentrations of PKC inhibitors did result in lower phospho-PKD levels. (Figure 18C) This result further shows that the 2 PKC inhibitors used have the ability to effiently inhibit PKC in B cells at the highest dose recommended (10 μM for Gö6976 and 5 μM of Ro-31-8220).

3.3.4.2. Inhibition of PKCs using Gö6976 and Ro-31-8220 reduced VLA-4 and LFA-1 integrin-mediated cell adhesion to fibronectin and ICAM-1.

After confirming that both PKC inhibitors were effective in inhibiting PKC at recommended concentrations, further static adhesion assays using fibronectin and ICAM-1 ligands were carried out to confirm that inhibition of PKC affected integrin-mediated cell adhesion.

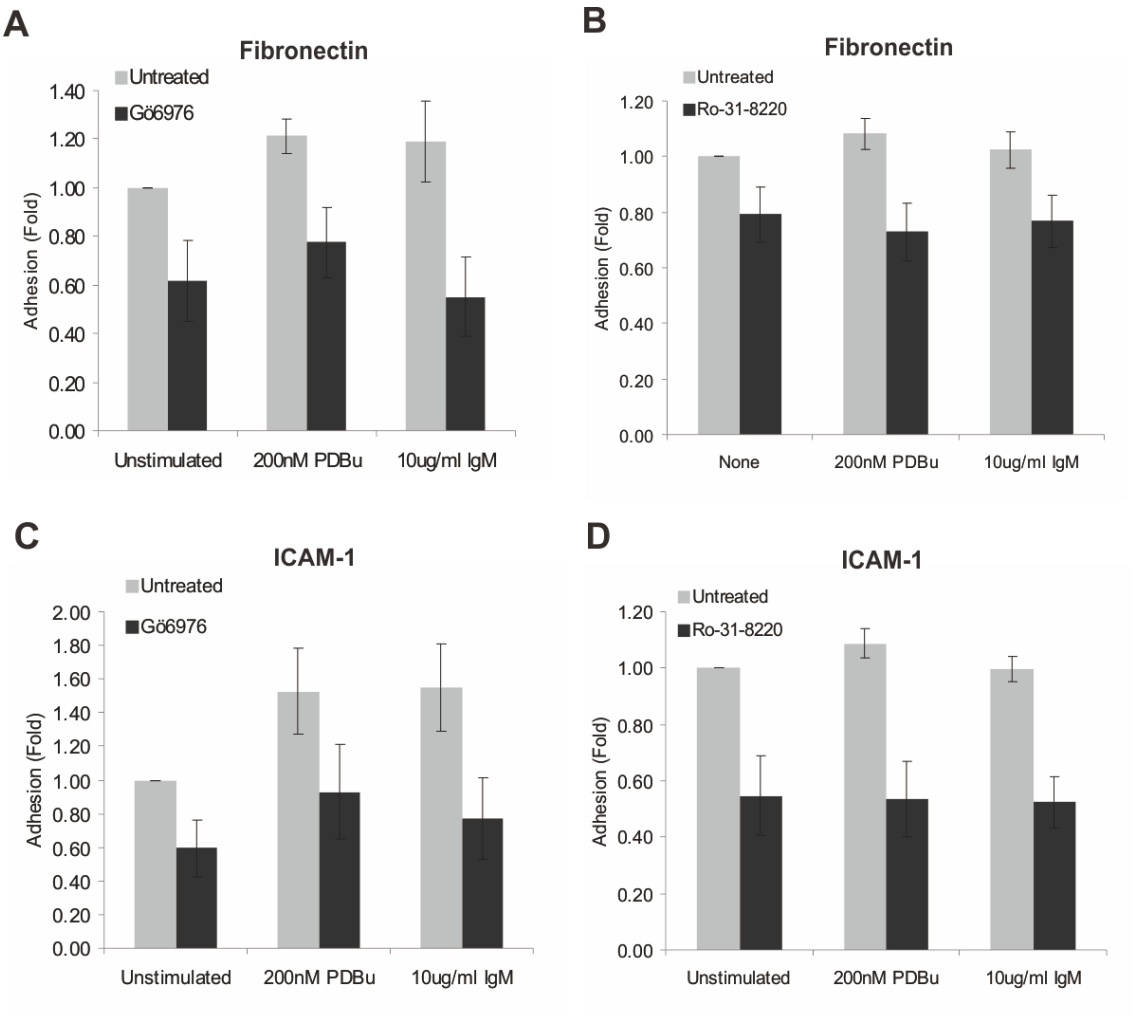


Figure 19. Inhibition of PKC kinase activity in B cells resulted in significantly lower cell adhesion to fibronectin and ICAM-1.

It was noted that stimulation of B cells with phorbol ester or through the BCR did not always significantly increase cell adhesion to ICAM-1, while stimulation of B cells did not significantly increase cell adhesion to fibronectin (Figure 19). Adhesion of total unstimulated B cells adhering to either fibronectin or ICAM-1 ranged from 18-35%. However in the assays comparing the effect of Gö6976 in B cells (Figure 19C), the percentage of total unstimulated cells adhering to ICAM-1 ranged from 24-68%, while 32-81% of phobol-ester stimulated B and 30-78% BCR-stimulated B cells adhered to ICAM-1, which are significant increase in cell adhesion to ICAM-1 compared to unstimulated cells. (Data not show). It appeared that cells could not be stimulated some times which could be due to high basal adhesion levels or biological variance between mice.

Significantly lower adhesion of B cells to fibronectin was observed when cells were treated with either Gö6976 or Ro-31-8220 and this implied that integrin-mediated cell adhesion (mediated by VLA-4 or other β1 integrins) was impaired when PKC was inhibited (Figure 19A and 19B). Similar results were observed when B cells were treated with either of the two PKC inhibitors and adhesion to ICAM-1 was measured, showing that LFA-1 integrin-mediated cell adhesion may also be dependent on PKCs (Figure 19C and 19D). However, as these inhibitors have the ability to inhibit various isoforms of PKCs, and are relatively unspecific, it was not possible at this point to establish which PKC isoform(s) (if any) were responsible for regulating integrin-mediated cell adhesion.

3.3.4.3. PKCβ, the main PKC isoform in B cells, is not important in regulating integrin-mediated cell adhesion

The major PKC isoform (PKCtheta) in T cells is involved in the regulation T cell receptor-stimulated integrin-mediated T cell adhesion. PKCβ is abundantly expressed in B cells and function similarly to PKCθ in T cells (Guo et al., 2004) but whether PKCβ controls cell adhesion and signalling is not clear. Therefore we decided to investigate if this PKC isoform is crucial for regulating integrin mediated B cell-adhesion. Enzastaurin is a bisindolymaleimide ATP competitive inhibitor that targets mainly PKCβ and was developed as a drug to treat Diffuse Large B cell lymphoma (Graff et al., 2005, Querfield et al., 2006). Enzastaurin was therefore used to assess its effect, if any, on B cell static adhesion (Figure 20).

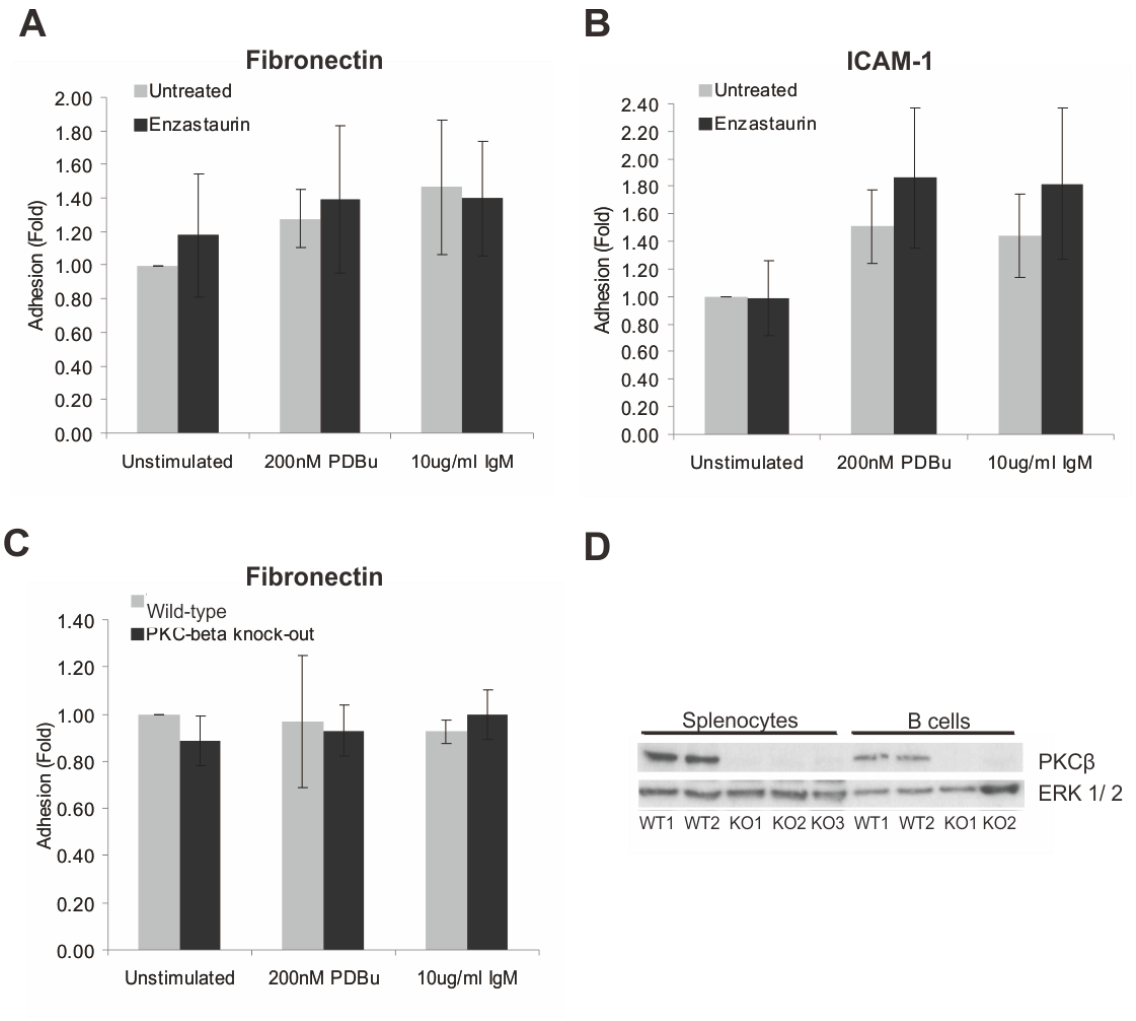


Figure 20. Inhibition of PKCβ and lack of PKCβ did not affect integrin-mediated cell adhesion.

(A) B cells from wild-type mice were pretreated with Enzastaurin (5 μM) and their adhesion to Fibronectin was measured. (B) Similar experiments were carried out using ICAM-1 ligands. For both (A) and (B), N=4 experiments. Inhibitor treatment did not affect cell adhesion to ligands, p>0.05, Student's t-test for experimental conditions. (C) Adhesion to fibronectin was measured using B cells isolated from wild-type mice or PKCβ knock-out mice. N=2. There were no significant differences between wild-type and PKCβ knock-out cells, p>0.05, Student's t-test for all experimental conditions. Error bars represent standard deviation for Figure A-C. (D) Western blot detection of PKCβ and total ERK1/2 from wild-type and PKCβ knock-out cells. N=2. N represents 1 experiment where cells were pooled from 2-3 mice.

It was noted that in these experiments, phorbol ester stimulation but not BCR stimulation could significantly increase B cell adhesion to ICAM-1, while stimulation of B cells with either of these agents did not significantly increase cell adhesion to fibronectin (Figure 20A-C). Adhesion % of unstimulated B cells adhering to either fibronectin or ICAM-1 ranged from 18-26%, while adherent phobol-ester stimulated B cells varied between 27-47% and adherent BCR stimulated cells ranged from 25%-42%. (Data not shown.) When B cells were treated with Enzastaurin, adhesion to the integrin ligand fibronectin and LFA-1 ligand ICAM-1 were not reduced when compared to cells not pre-treated with the inhibitor. These results from static adhesion assays using Enzastaurin suggested that PKCβ was not crucial in regulating integrin-mediated B cell adhesion.

To confirm these results, PKCβ knock-out mice were obtained from Michael Leitges' laboratory in Oslo. Experiments with PKCβ knock-out B cells showed that deleting PKCβ (as shown here by control experiments detecting PKCβ by Western blotting from wild-type and PKCβ knock-out B cells, Figure 20D) did not cause a significant difference in adhesion to fibronectin when compared with wild-type cells. There was also no significant difference observed whether the B cells were non-stimulated or stimulated by phorbol ester or through the BCR (Figure 20C). This result was therefore similar to that using wild-type B cells pre-treated with Enzastaurin (Figure 20A). Flow cytometry analysis using wild-type and PKCβ knock-out B cells showed that these cells had similar levels of integrins on the cell surface, showing that surface expression of integrins were also not affected by knocking out PKCβ. (Experiment done by Sharon Matthews.) These results showed that the lack of the most abundant PKC isoform in B cells, PKCβ did not affect cell adhesion. Therefore, it is possible that other PKC

isoforms or other downstream effectors of PKC could compensate for this loss of function of PKCβ.

3.3.4.4. PKD, which is downstream of PKC, is not important in the regulation of integrin-mediated cell adhesion in splenocytes

As mentioned in the previous sections and Figure 6C, PKD (also known as PKCμ) is downstream of PKC. PKD is regulated by a PKC-dependent pathway through DAG (diacylglycerol) (Matthews et al., 2010). PKC isoforms and their downstream effector, PKD have been previously implicated in lymphocyte integrin regulation (Medeiros et al., 2005). To investigate the putative role of PKD in B cell adhesion downstream of PKC isoforms, a novel specific PKD inhibitor (Meredith et al., 2010), Novartis 12a was selected to assess whether integrin-mediated B cell adhesion was impaired when PKD kinase activity was inhibited (Figure 20).

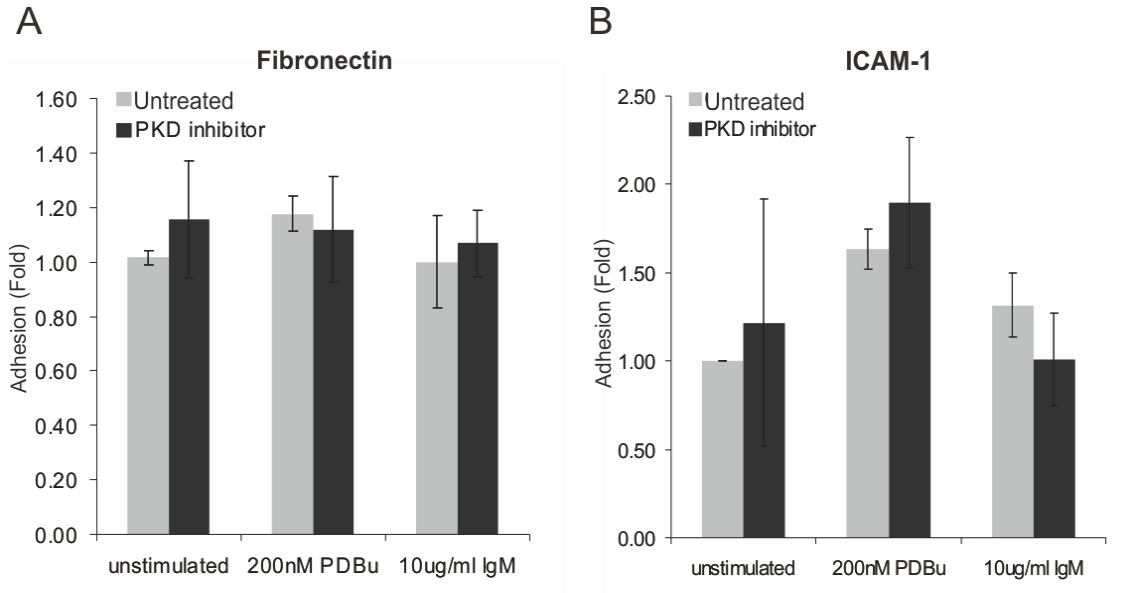


Figure 21. PKD inhibition in splenocytes did not affect integrin-mediated cell adhesion.

(A) Splenocyte adhesion to fibronectin was assessed, comparing untreated cells and cells pre-treated with a PKD inhibitor (Novartis 12a at 5 μM) (B) Similar experiments were performed to assess adhesion of splenocytes to ICAM-1 with or without pre-treatment of PKD inhibitor (Novartis 12a). N=3 experiments, with 1 mouse per experiment. P>0.05, Student's t-test. Error bars represent standard deviation. Experiments were carried out together by Susanna Fagerholm, Vicky Morrison and Hwee San Lek.

The percentage and fold increase of non-inhibitor treated adhered splenocytes in Figure 21 is the same as Figure 15 as these are from the same set of experiments, which is 5-6% of unstimulated splenocytes adhering to both ICAM-1 and fibronectin while 8-10% of phobol ester stimulated splenocytes adhered to ICAM-1 (as discussed earlier). Splenocytes were obtained from wild-type mice and pre-treated with Novartis 12a to assess if B cells require PKD for integrin-mediated cell adhesion. Splenocytes did not show any significant reduction in adhesion to the fibronectin following pre-treatment of cells with the PKD inhibitor. This was irrespective of whether the cells were unstimulated or stimulated with phorbol ester or through the BCR (Figure 21A). Similar experiments performed using the LFA-1 ligand ICAM-1 in the adhesion assay also showed that there was no significant difference in cell adhesion, regardless of stimulation, between cells which were pretreated with the PKD inhibitor or untreated cells (Figure 21B). These results indicated that PKD (inhibited using Novartis 12a) does not play a significant role in B cell integrin-mediated static adhesion. Positive controls with inhibitors that are known to reduce cell adhesion were not carried out due to lack of cell samples. Western blot analyses (carried out by Sharon Matthews) has also shown that increasing concentration of Novartis 12a PKD inhibitor can reduce phospho-PKD in effector cycotoxic T cells while static adhesion assays showed that unstimulated or stimulated effector cytotoxic T cells pre-treated with Novartis 12a PKD inhibitor did

not display a significant decrease in cell adhesion to fibronectin or ICAM-1. (Carried out by Hwee San Lek. Data not shown.)

There are 3 isoforms of PKD – PKD1, PKD2 and PKD3. PKD1 is not expressed in murine lymphocytes while some PKD3 is expressed. PKD2 however, is the most abundant PKD isoforms in murine lymphocytes (Matthews et al., 2010). The inhibitor, Novartis 12a, is able to inhibit all PKD isoforms (Meridith et al., 2010). In order to confirm that the lack of PKD2 did not affect integrin-mediated cell adhesion, B cells were isolated from wild-type and PKD2 knock-out and PKD2 knock-in mice and their adhesion to integrin ligands was assessed (Figure 22). PKD2 knock-out and PKD2 knock-in mice were produced and provided by Doreen Cantrell (University of Dundee). PKD2 knock-out cells did not express PKD2 while PKD2 knock-ins had truncated, non-functional PKD2 (Matthews et al., 2012).

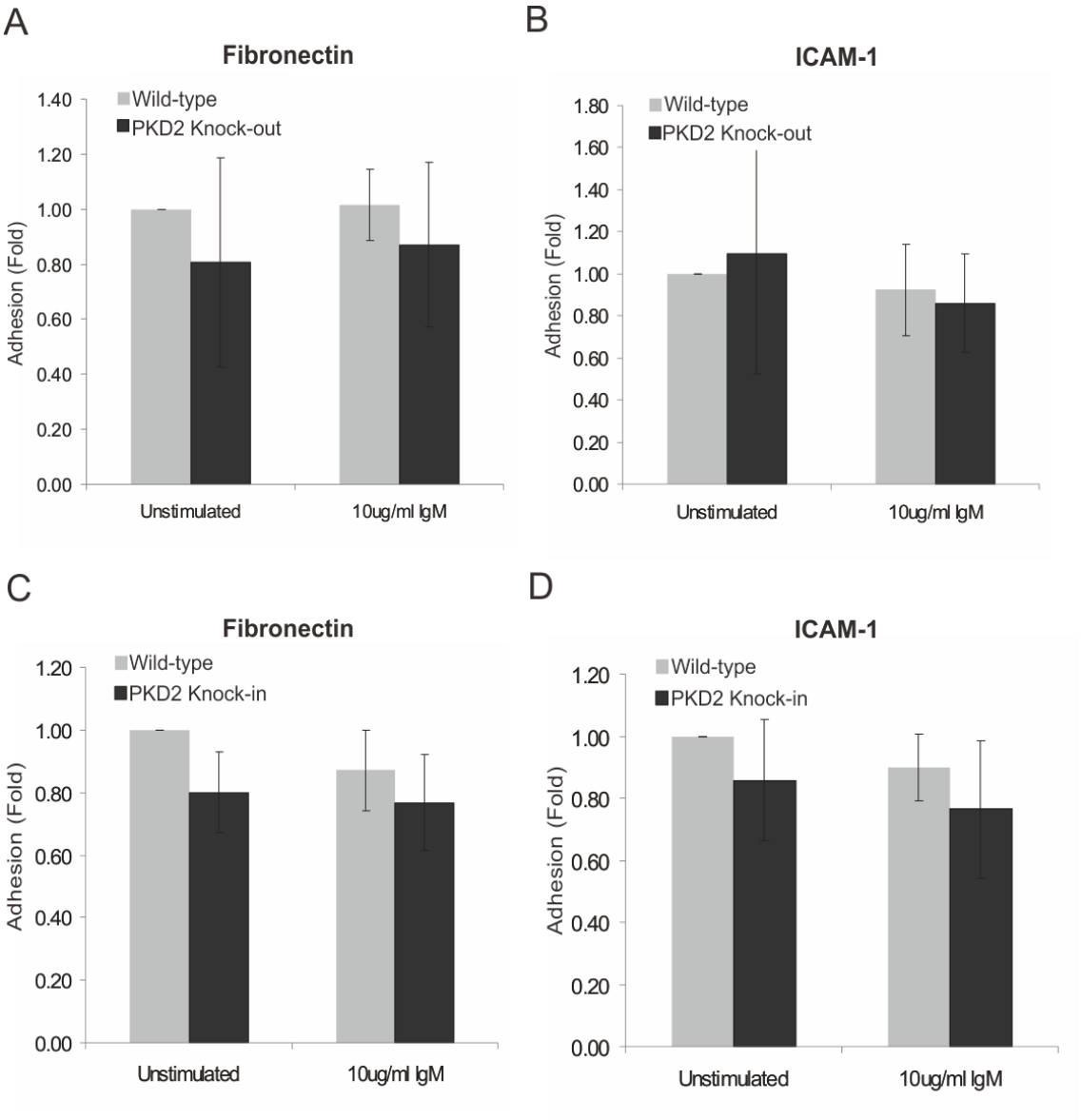


Figure 22. B cells lacking PKD2 showed no significant reduction in integrin-mediated cell adhesion.

(A) Adhesion of B cells purified from PKD2 knock-out mice or wild-type mice to fibronectin was measured. Cells were left unstimulated or stimulated at the BCR using IgM and left to adhere on fibronectin. (B) Similar adhesion assays as in (A) were carried out using ICAM-1 ligands. (C, D) B cells purified from PKD2 knock-in mice and wild-type mice were obtained and adhesion to fibronectin (C) or ICAM-1 (D) was measured. N=4 pairs of mice, $p>0.05$, Student's T-test for all experimental conditions. Error bars represent standard deviation.

BCR stimulation of B cells from wild-type, PKD2 knock-in and PKD2 knock-out mice did not significantly increase adhesion to fibronectin and ICAM-1 ligands. Amount of unstimulated wild-type cell adhering to fibronectin ranged from 14%-26% and 15-34% on ICAM-1 (Figure 22). B cells lacking PKD2 did not have impaired adhesion to fibronectin (Figure 22A) or ICAM-1 (Figure 22B). There was no significant difference in adhesion whether the B cells were unstimulated or stimulated at the BCR (Figure 22A and 22B). B cells that did not have functional PKD2 were also not significantly different in integrin-mediated cell adhesion to fibronectin (Figure 22C) or ICAM-1 (Figure 22D). Flow cytometry analysis to analyse cell surface expression of integrins in PKD2 knock-in and PKD2 knock-out B cells was also carried out and there was no significant difference in integrin surface expression in these cells when compared to wild-type B cells. (Flow cytometry carried out by Sharon Matthews, data not shown). These results show that the lack of PKD2 or the presence of functional PKD2 was not necessary for expression of integrins at the cell surface or for cell adhesion.

3.3.4.5. RSK does not play a role in regulation of integrin-mediated adhesion to fibronectin but may play a role in the regulation of the LFA-1 integrin in B cells.

As we were not successful in determining if PKC was important for B cell adhesion and if so, which isoform in the PKC family of kinases was responsible for controlling integrin-mediated B cell adhesion, the focus of the project was shifted to investigate if other members in the AGC kinase family, such as RSK and/or Akt played a role in B cell adhesion. The role of AGC kinases in integrin regulation in lymphocytes has not previously been extensively investigated (Figure 23).

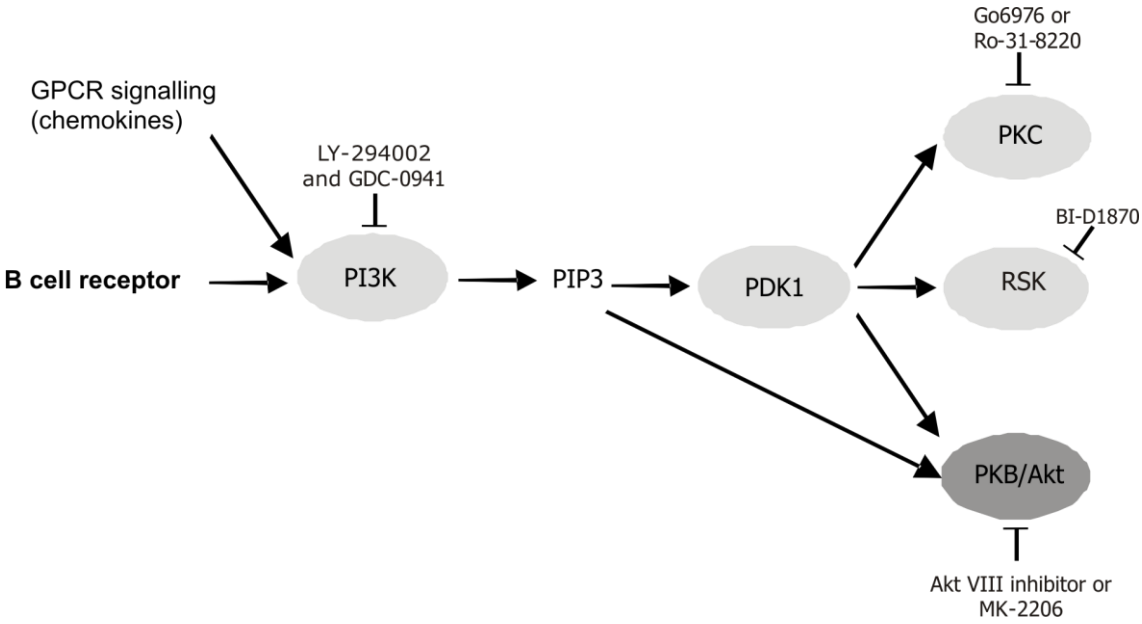


Figure 23. Schematic figure of BCR-induced PI3K/PDK1 signalling pathways and inhibitors used to target specific kinases (PI3K, PKC, RSK and Akt) in this study.

PDK1 activates RSK which is also known as p90 ribosomal S6 kinase (Mora et al., 2004). We started by investigating the role of RSK in B cell adhesion. The RSK inhibitor used in this study was BI-D1870. This is an ATP-competitive inhibitor of the N-terminal AGC kinase domain of RSK (Sapkota et al., 2007), which has been tested previously. B cells were isolated and adhesion assays were carried out to assess if inhibition of RSK would affect integrin mediated cell adhesion and specifically LFA-1 integrin mediated cell adhesion (Figure 23).

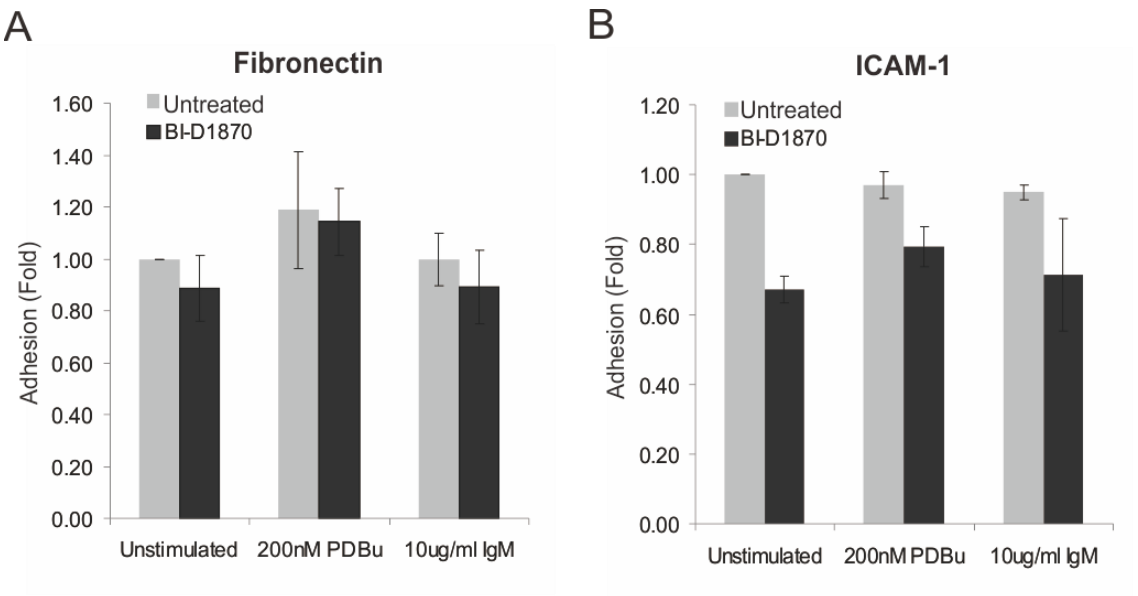


Figure 24. Adhesion of B cells to fibronectin was unaffected by RSK inhibition but RSK inhibition affects B cell adhesion to ICAM-1.

(A) B cells from wild-type mice were pretreated with the RSK inhibitor (20μM) and the adhesion to fibronectin under unstimulated, phorbol ester stimulated or BCR triggered conditions was measured. N = 4 experiments, p>0.05, Student's t-test for both unstimulated B cells and stimulated B cells. (B) A similar assay was carried out using ICAM-1 ligands. N= 3 experiments, p<0.05, Student's t-test for unstimulated B cells but p>0.05, Student's t-test for phorbol ester stimulated and BCR-triggered B cells. Error bars indicate standard deviation. Each experiment required mice B cells pooled from 2-3 mice.

Phobol ester and BCR stimulation of B cells did not significantly increase adhesion to fibronectin and ICAM-1 ligands. Amount of unstimulated B cell adhering to fibronectin ranged from 15%-32% and 28-32% on ICAM-1 (Figure 24). B cells pre-treated with the RSK inhibitor BI-D1870 did not display any significant decrease in cell adhesion to fibronectin, regardless of whether the cells were unstimulated or stimulated (Figure 24A). However, unstimulated, B cells pre-treated with the RSK inhibitor had significantly lower adhesion to ICAM-1 ligand than untreated B cells. However, phorbol ester-treated or BCR stimulated cell adhesion to ICAM-1 was not affected by

the RSK inhibitor (Figure 24B). Taken together, RSK may have an effect in regulation of cell adhesion in unstimulated B cells but stimulation of B cells through the BCR or with phorbol ester may activate other kinases that override the role of RSK activity in integrin regulation. In future, Active RSK levels in cell lysates should also be checked using western blot detection to confirm that the inhibitor is effective.

3.3.4.6. The PI3K/Akt pathway regulates integrin-mediated cell adhesion in B cells.

The role of RSK in adhesion was not obvious from the results in the static adhesion assays. We therefore decided to investigate if other major AGC kinases control integrin-mediated cell adhesion in B cells. PI3K, which is upstream of AGC kinases, has been shown to be important in B cell integrin activation and in B cell homing (Nombela-Arrieta et al., 2004, Arana et al., 2008) (Figure 23).

LY294002 and GDC-0941 are two commonly used PI3K inhibitors that can be used to study the effects of PI3K in cells and these were chosen to assess the role of PI3K in integrin-mediated B cell-adhesion. These inhibitors are both ATP-competitive inhibitors (Vlahos et.al., 1993, Folkes et al., 2008). We decided not to use wortmannin as it was originally used as an inhibitor of SmMLCK and has been shown to be highly unspecific in inhibition of PI3Kinase superfamily and protein kinases (Davies et al., 2000). LY294002 was chosen as it is also a more stable inhibitor that wortmannin when in solution and the recommended dosage was 10µM-50µM. Although LY294002 can inhibit Class 1 PI 3kinase, it also has off target effects such as TORC1, CK2, PLK1, GSK3, PIM1, PIM3, HIPK2 and other ATP-binding proteins which are not protein

kinases. Due to such non-specificity, it was also recommended not use this inhibitor (Bain et al., 2007). However, this inhibitor has been widely used and published, and we therefore decided to also use another structurally different PI 3 kinase inhibitor to ensure that the effects observed in assays are not due to off-target effects, as suggested by Davies et al. (2000) when choosing inhibitors. The other PI3K inhibitor chosen was GDC-0941. GDC-0941 is a relatively new inhibitor which has been approved for clinical trials in 2008 for its high bioavailability and anti-tumour effect. This inhibitor is a specific Class I PI 3 kinase isoforms inhibitor which is able to inhibit all 4 isoforms at 1µM but has little or no effect on other classes of PI 3 kinase (Kong et al., 2010).

Another AGC kinase of interest in relation to the current study is the Akt family, which consists of 3 isotypes: Akt1, Akt2 and Akt3. Activation of PDK1 (the master regulator of AGC kinases) can activate Akt (Bain et al., 2007). Akt is also known as PKB or RAC-protein kinase and is the most studied downstream target of PI3K (Vanhaesebroeck and Alessi, 2000). In addition to the PI 3-kinase inhibitors described above, we used Akt inhibitors (Akt VIII inhibitor and MK2206), to investigate the putative role of the PI3K/Akt pathway in the regulation of B cell adhesion. The AktI-1/2 inhibitor (also known at Akt VIII inhibitor) was recommended by Bain et al., (2007). At concentrations as low as 1µM, this inhibitor is highly specific and can inhibit Akt1 or CaMK1 (Calcium/calmodulin-dependent protein kinase type 1) by 80% without affecting other protein kinase tested. This inhibitor works by binding to the PH domain, preventing the initial conformational changes triggered by PIP_3 binding which would allow activation of Akt phosphorylation loop at Thr308 by PDK1 (Bain et al., 2007). MK2206 is also used as a new chemotherapy drug to treat leukaemia. Also the MK2206 inhibitor targets the Akt Pleckstrin Homology domain and is an allosteric inhibitor (Li et al., 2009).

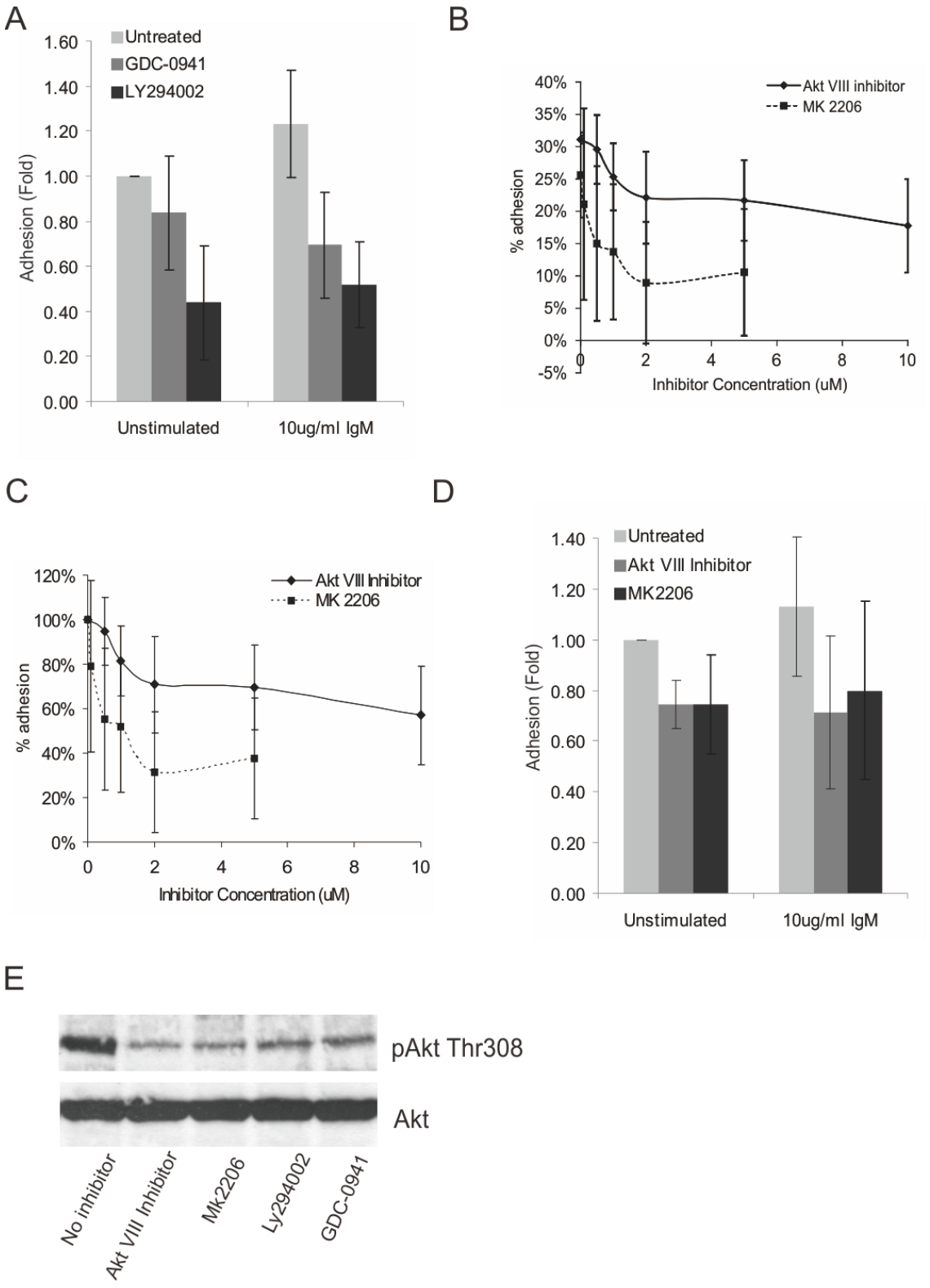


Figure 25. Adhesion of B cells to ICAM-1 is reduced by PI3K/Akt inhibition.

(A) B cells from wild-type mice were pretreated with LY294002 (50 μM) or GDC-0941 (1 μM) and their adhesion to ICAM-1 was assessed. Cells were left unstimulated or stimulated at the

BCR using IgM. N=3-7 for unstimulated cells. N=7-12 for BCR stimulated cells. P<0.05, Student's t-test for both inhibitor treatments. (B) Dosage curve showing actual B cell adhesion (in percentages) to ICAM-1 under the effect of Akt inhibitors, Akt VIII and MK2206, stimulated at the BCR using IgM. N=3 experiments. (C) Data from (B) presented as percentage decrease after inhibitor treatment, where cells without inhibitor treatment adhering to ICAM-1 was assigned as 100%. (D) B cells were pretreated with Akt VIII (10μM) or MK2206 (5 μM) and their adhesion to ICAM-1 assessed. N=3-7 experiments. p<0.05, Student's t-test. In all graphs, error bars indicate S.D. (E) Western blot detection of active phosphorylated Akt 308 (pAkt) and total Akt levels in untreated, Akt VIII inhibitor-treated (10 μM), MK2206-treated (5 μM), LY294002-treated (50 μM) and GDC-0941-treated (1 μM) BCR-stimulated B cells. The result shown is representative of N=3 experiments. N numbers represent the number of experiments in which spleens from 2-3 mice were pooled together for each experiment. The treatment time for GDC-0941, LY294002 and Akt VII inhibitor was 30minutes and 1 hour for MK2206.

BCR stimulation of B cells did not significantly increase adhesion to ICAM-1 ligands. The amount of unstimulated B cell adhesion to ICAM-1 ranged from 18%-89% on ICAM-1 and therefore, the results were presented as fold-change to take into consideration the large biological variability between mice (Figure 25). B cells pre-treated with PI 3-kinase inhibitors, GDC-0941 or LY290002, had significantly reduced adhesion to ICAM-1 under non-stimulated and BCR stimulated conditions (Figure 25A). Akt VIII was recommended to be used at 10 μM to pre-treat cells for 30 minutes by the manufacturer, Merck. The effect of the inhibitor could be seen when it was used at a concentration as low as 0.5 μM and a good response could be observed when the inhibitor was used at 2 μM. Bain et al. (2007) recommended the use of Akt 1/2 inbibitor at 1μM which should be checked in adhesion assays and western blot detection of active Akt levels in future.. Like the Akt VIII inhibitor, the effect of MK2206 on cell adhesion to ICAM-1 could also be observed from as low a concentration as 0.1 μM (Figure 25B

and 25C). The recommended dosage of 5 μM MK2206 was very effective in reducing B cell-adhesion to the ICAM-1 ligand (Figure 25D). It was recommended by the manufacturers to allow for an hour when pre-treating cells with MK2206, instead of 30minutes.

Western blot analysis showed lower levels of active phosphorylated Akt in B cells pre-treated with Akt inhibitors (Akt VIII inhibitor and MK2206), or PI3K inhibitors (LY2940002 and GDC-0941) when cells were stimulated through the BCR with IgM, when compared to B cells without inhibitor pre-treatment, showing the effectiveness of these inhibitors in inhibiting the PI3K/Akt pathway in murine B cells. Western blot detection of total Akt levels was used as a control for protein loading (Figure 25E). Phospho-Akt (Thr308) was also observed to be decreased in unstimulated mouse B cells after inhibitor treatment. (Data not shown.) In summary, these results showed that the PI3K and Akt inhibitors were effective and both Akt VIII inhibitor and MK2206 pre-treatment of murine B cells significantly reduced integrin-mediated B cell static adhesion to ICAM-1 (Figure 25).

3.3.5. Adhesion of $CD4^+$ T cells to ICAM-1 is affected by PKC inhibition but not by Akt inhibition.

After the investigations on the roles of AGC kinases in B cell adhesion, it was important to investigate if $CD4^+$ T cells were dependent on PKC and/or P I3-kinase/Akt pathway for adhesion. $CD4^+$ T cells were purified from spleens and lymph nodes, pre-treated with PKC inhibitors Ro-31-8220 or with Akt inhibitor, Akt Inhibitor VIII and their adhesion to ICAM-1 was assessed.

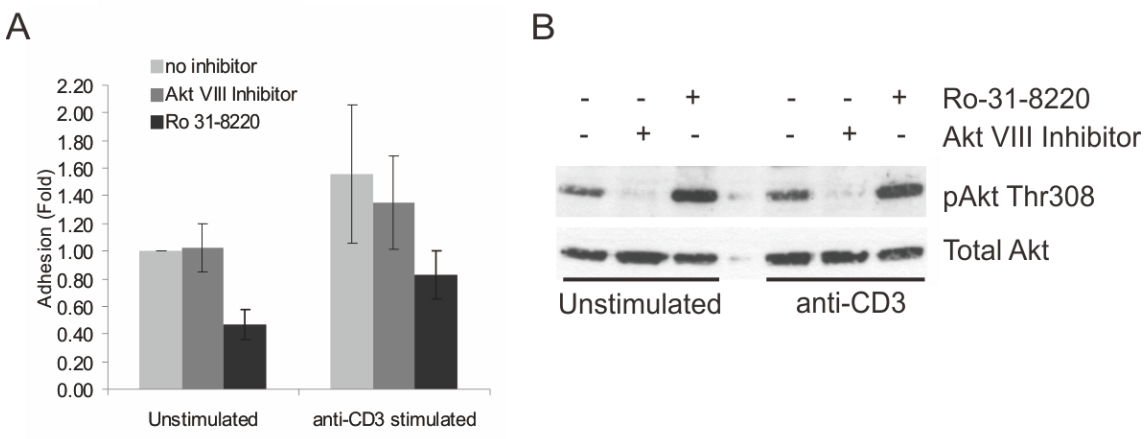


Figure 26. Adhesion of CD4⁺ T cells to ICAM-1 is not reduced by Akt inhibition but is affected by PKC inhibition.

*(*A) Effect of Akt VIII inhibitor (10 μM) and PKC inhibitor Ro-31-8220 (5 μM) treatment on $CD4^+$ T cell adhesion to ICAM-1. Cells were either unstimulated or TCR-triggered. The Akt VIII inhibitor treatment data is pooled from 6 experiments, $p<0.05$, Student's t-test for TCR-triggered $CD4^+$ T cells. The PKC inhibitor data is pooled from 4 experiments, $p<0.05$, Student's t-test. In all graphs, error bars indicate S.D. (B) Western blot detection of levels of phosphorylated Akt 308 (pAkt) levels and total Akt levels in Ro-31-8220 or Akt VIII inhibitor treated, anti-CD3-stimulated $CD4^+$ T cells. *Spleens and lymph nodes from 2-3 mice were pooled together for each experiment.*

Although TCR stimulation of $CD4^+$ T cells led to an increase in cell adhesion to ICAM-1, this increase was not statistically significant (N=6 experiments). Unstimulated T cell adhesion to ICAM-1 ranged from 21%-59% on ICAM-1 and adhesion of anti-CD3 stimulated $CD4^+$ T cells ranged from 25%-90% (Figure 26). Unlike the situation in B cells (Figure 25), pre-treatment of $CD4^+$ T cells with the Akt VIII inhibitor did not significantly affect cell adhesion to ICAM-1. In contrast, $CD4^+$ T cells pre-treated with PKC inhibitor Ro-31-8220 were significantly less adhesive than control cells. This suggested that LFA-1 in $CD4^+$ T cells was more likely to be regulated by PKC instead

of Akt, whereas Akt and PKC (but not PKCβ) was needed in B cells to regulate LFA-1 integrin activation (Figure 19, 20 and 26A). The Akt VIII inhibitor was effective in reducing active phospho-Akt 308 levels in $CD4^+$ T cells even though cell adhesion was not affected. In addition, Ro-31-8220 treatment of $CD4^+$ T cells slightly increased phosphorylated Akt levels (Figure 26B). It was demonstrated by other researchers that inhibition of novel PKC isoforms (PKCε) using Ro-31-8220 can increase phosphorylated Akt levels and overexpression of PKCε can suppress active Akt activation (Wen et al., 2002, Liu et al., 2006). Although those experiments were carried out in cell lines, this could explain our observations in Figure 26B.

3.3.6. PKC regulates Rap1 activation in B cells

Rap1 has been found to regulate the LFA-1 integrin in T cells (Katagiri et al., 2000, Letshchka et. a., 2008). Rap1b is also important for B cell development in bone marrow and marginal zone homeostasis (Chu et al., 2008) and deletion of Rap1b leads to defects in short term homing of B cells to lymph nodes (Chen et al., 2008, Chu et al., 2008). PKCθ, the major PKC isoform in T cells was found to regulate integrin activation via Rap1. Therefore, we decided to investigate whether AGC kinases, especially PKC, were involved upstream in regulating Rap1 activation in murine B cells. The effect of AGC kinase inhibitors on Rap1 activity was analysed using an Active Rap1 pull-down assay kit (Pierce). This kit uses a fusion protein of the Rap1-binding domain (RBD) from human Ral guanine dissociation stimulator protein, GST-RalGDS-RBD, which binds the GTP-form of Rap1 to detect the active form of the small GTPase Rap1.

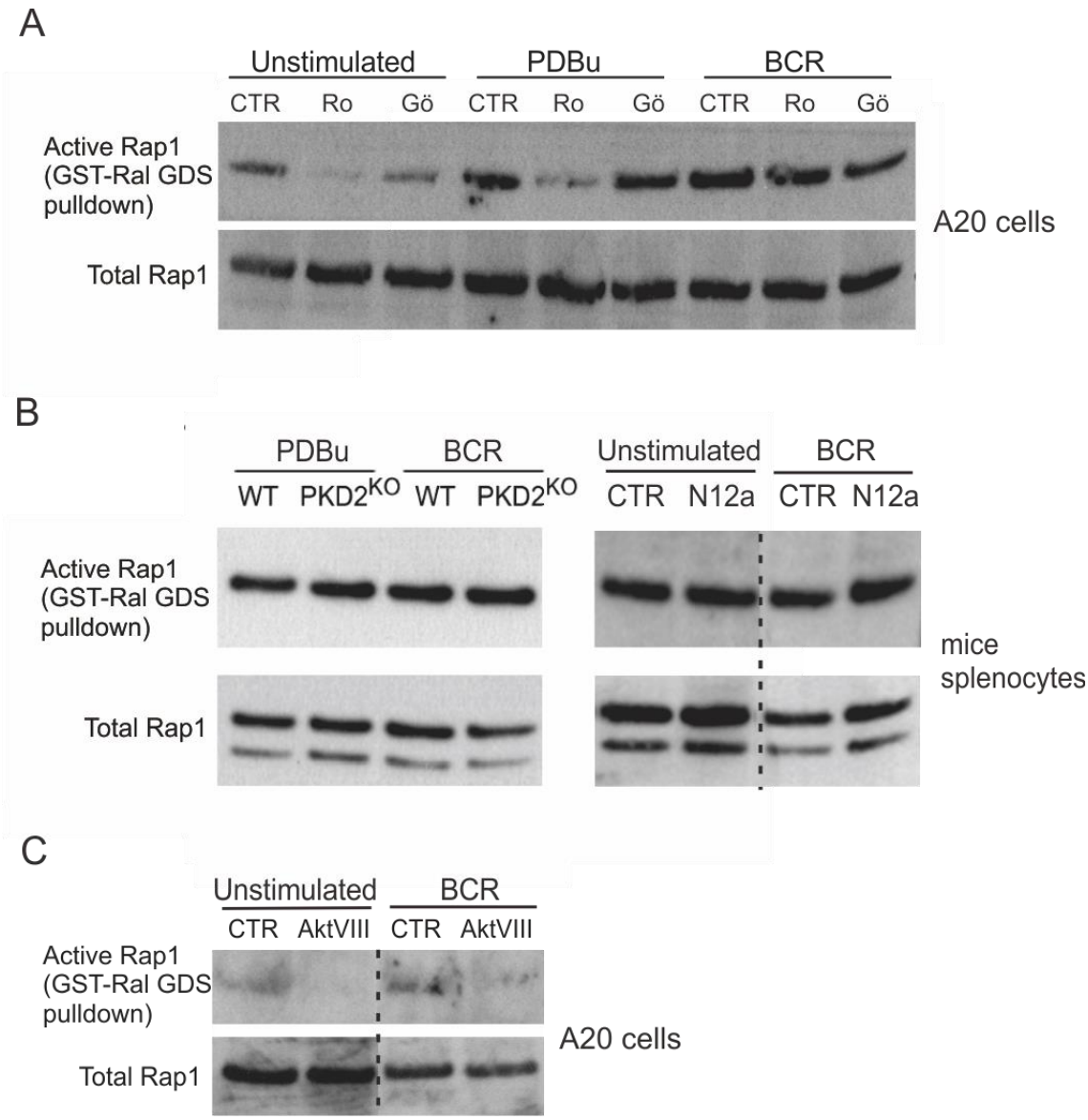


Figure 27. Detection of downstream Rap1 levels in B cells.

(A) Active Rap1 levels in PKC inhibitor-treated B cell line A20. Cells were treated with 5 μM Ro-31-8220 or 10 μM Gö6976 and then left unstimulated, or stimulated by phorbol ester or at the B cell receptor. A representative Western blot from N = 2 experiment is shown. (B, left) Western blot detection of active and total Rap1 levels in wild-type versus PKD2 knock-out splenocytes, stimulated by phorbol ester or at the B cell receptor. (B, right) Western blot detection of levels of active Rap1 in splenocytes from wild-type mice untreated or pretreated with Novartis 12a PKD inhibitor, stimulated by phorbol ester or at the B cell receptor. A representative Western blot from N=3 experiments is shown. (C) Western blot detection of

active Rap1 levels and total Rap1 levels in A20 cells pre-treated with Akt VIII inhibitor. A20 cells were left unstimulated or stimulated at B cell receptor. A representative western blot from N=3 experiments is shown..

As PKC inhibitors reduced B cell receptor and phorbol ester induced B cell adhesion, the levels of active Rap1 in inhibitor-treated cells was assessed using A20 cells, a mouse lymphoma B cell line. A20 cells were used instead of primary splenic B cells as this method provided more protein in cell lysates for the assay. Interestingly, both Ro-31-8220 and Gö6978 decreased active Rap1 levels when compared to the untreated cells in unstimulated and phorbol ester stimulated conditions, but not in B cell receptor stimulated cells. Ro-31-8220 treated cells had less active Rap1 than Gö6978 treated cells, which could be due to the ability of Ro-31-8220 to inhibit more PKC isotypes than Gö6978, which inhibits mainly classical PKCs. Rap1 activity in resting and phorbol ester stimulated B cells was affected by PKC-inhibition, implicating that Rap1 may indeed be the downstream effector of PKC in integrin activation under these conditions (Figure 27A).

However, A20 cells stimulated at the B cell receptor and pre-treated with PKC inhibitors did not display a significant decrease in active Rap1 levels. Considering the results shown in Figure 19 and 27A, although B cell receptor stimulated B cells pre-treated with PKC inhibitors had significantly less adhesion to ICAM-1, the Rap1 activity in B cells was not affected. This suggested that BCR-activated PKC isotypes may not influence integrin activation through Rap1, but instead through some other mechanism.

3.3.7. Lymphocytes lacking PKD2 displayed normal adhesion and normal activation of Rap1 when compared to wild-type cells.

PKD is a downstream effector of PKC in cells, and has been previously implicated in lymphocyte integrin regulation through Rap1 (Medeiros et al., 2005). Therefore, we wished to determine whether this kinase plays a role in Rap1 regulation in primary murine B cells. Both wild-type and PKD2 knock-out splenic B cells had similar levels of active Rap1, indicating that, in vivo in murine B cells, PKD2 did not play a role in Rap1 activation. Similar experiments using wild-type mice splenic B cells, comparing the levels of active Rap1 with and without PKD inhibitor, Novartis 12a also showed no decrease in Rap1 activity. Similar results were observed in resting or stimulated conditions. Together with adhesion data in Figure 20, these results showed that integrin-mediated cell adhesion of B cells lacking PKD was unaffected as PKD did not affect Rap1 activation in primary B cells (Figure 27B).

3.3.8. Akt VIII inhibitors reduced Rap1 activation in B cells.

To investigate if Akt also regulates Rap1 in B cells, we investigated Rap1 activation in A20 cells. Akt VIII inhibitor pre-treated B cells had reduced active Rap1 when unstimulated and also when their B cell receptor was triggered, indicating that Akt could indeed regulate B cell adhesion through Rap1 (Figure 27C). Taken together, these results indicated that although some PKC isoforms regulate integrin-mediated B cell adhesion through both Rap1-dependent and independent mechanisms, PI3K/Akt signalling which leads to the activation of Rap1 has a significant effect in activating

integrins when B cells are stimulated at the B cell receptor. Results from the B cell studies thus far are summarised below:

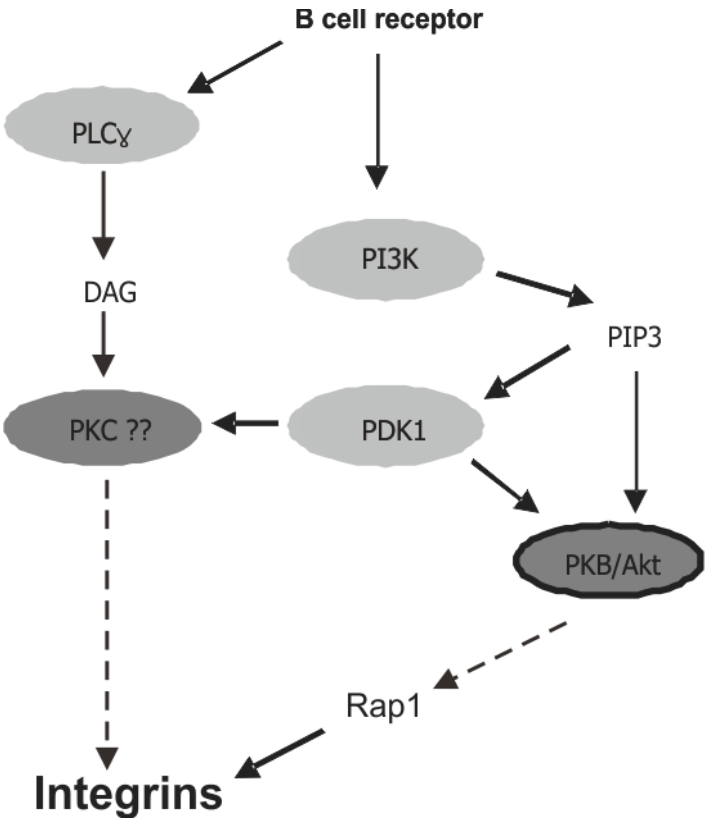


Figure 28. Schematic summary of signalling pathways regulating integrins in B cells: focus on Akt and PKC signaling and the involvement of Rap1.

B cell receptor triggering can also lead to the activation of the Class IA p110δ PI3K/Akt signalling pathway. Increased PI3K-mediated production of PIP3 activates Akt by conformational changes. PIP3 also promotes PDK1 localisation to the cell membrane, where phospholipid binding and activation loop phosphorylation at Thr308 of Akt occurs. Phosphorylation within the carboxyl terminus at Ser473 of Akt is needed for maximum activation of Akt (Sato et al., 2002, Scheid and Woodgett, 2003). Results from Figure 26 suggest that Akt has a role in regulation of Rap1 which may activate LFA-1 integrin binding to ICAM-1. However, it is uncertain if PKC is involved and if so, which PKC isoform regulates integrin-mediated cell adhesion in BCR-stimulated B cells, and what the downstream effector in integrin regulation is in this pathway .

3.3.9. Development of physiological relevant methods to study lymphocyte adhesion and migration under shear flow conditions

The static adhesion assay method to investigate the adhesive properties of integrins on lymphocytes makes use of gravity to gently "wash off" cells from ligands and is generally static and without the influence of any external forces. The static adhesion assays mimics the following situations: cell adhesion between two cells such as in antigen presentation or T cell priming, or interstitial migration of B cells in the spleen and T lymphocytes in lymph nodes. In the physiological setting of blood flow, cells are exposed to shear stress due to hemodynamic forces along the vascular endothelium and this could have a big impact on integrin-mediated adhesion.

Shear stress can range from 0.5-6 dynes/cm^2 in veins, to 16 dynes/cm^2 in venules and up to 70 dynes/cm^2 in arteries depending on the proximity to the heart and type of blood vessels (Papaioannou and Stefandis, 2005) Leukocytes adhesion occurs when the shear stress is lower than 4 dynes/cm^2 (Fung, Biomechanics, 1997). Shear stress influences cell behaviour in various ways. The presence of shear stress activates PKCε regulation of ERK1/2 responsible for gene transcription, cell proliferation and protein synthesis in endothelial cells (Paszakowiak and Dardik, 2003). Shear stress is also important in the upregulation of ICAM-1, E-selectins and VCAMs on endothelial cells, which increases lymphocyte adhesion (Nagel et al., 1993, Papaioannou and Stefandis, 2005). It was shown that high and low shear stresses differentially regulate the expression of VCAM-1 and ICAM-1 on endothelial cells. Under low shear stress-conditions of 3 dynes/cm2, there is much higher expression of VCAM-1 and lower ICAM-1 expression which favours monocyte adhesion (Wapola et al., 1995, Traub and Berk, 1998).

Mechanical forces generated by shear stress are thought to be detected and transformed into biological signals described as the decentralised model where shear stress acting on the cell surface are transmitted through the cytoskeleton, allowing the activation of integrins connected to the cytoskeleton (Papaioannou and Stefandis, 2005). Evidence suggests that the presence of shear force can enhance adhesion in T cells and peripheral blood lymphocytes via integrin binding, L-selectin interactions, shear force induced mechanical signals and G_i protein-mediated chemokine signalling (Cinamon et al., 2001, Woolf et al., 2007). Therefore, development of leukocyte adhesion assays with the introduction of shear stress was necessary to investigate adhesion in conditions mimicking the conditions in a blood vessel. Such shear flow assays would also enable us to observe cell processes such as rolling, spreading and extravasation.

In static adhesion assays, mouse B cells and B cell lines were stimulated using phorbol ester or by triggering the B cell receptor using antibodies. Shear flow adhesion assays were set up using IBIDI VI 0.4 chamber slides with immobilized ICAM-1, attached to a syringe pump which controls the volume of cell suspension to be dispensed over time, generating the required shear stress in the chambers. The cell suspension consisted of mouse splenic B cells pre-treated and stimulated as per static adhesion assays. With the introduction of shear stress, we could assess if adhesion under shear flow was regulated by similar signalling pathways as under static conditions.

However, lymphocytes in blood vessels are not stimulated at the B cell receptor in vivo but are stimulated by chemokines at the G-protein coupled receptors activating p110γ Class IB PI3K, which leads to adhesion to blood vessel walls followed by extravasation from blood vessels into lymph nodes or sites of inflammation (Werner et. al., 2010, Limon and Fruman 2012). Shear flow adhesion assays were therefore set up using SDF-

1 (CXCL12), a chemokine that is released onto the ICAM-1 expressing endothelium to encourage B cells extravasation. By coating SDF-1 on top of ICAM-1, this mimics sites for ligand binding which enables arrest of lymphocytes under shear flow (Shamri et al., 2005). SDF-1 was chosen as it has been well-studied and it is a chemoattractant for B and T cells, DCs and monocytes /macrophages, which binds to CXCR4 which is highly expressed on these immune cell types (Weiss et al., 2013). CXCL13 (a B cell chemoattractant) which binds to CXCR5 can also be used for assays specifically studying B cells (Guinoa et al., 2011, Rupprecht et al., 2009).

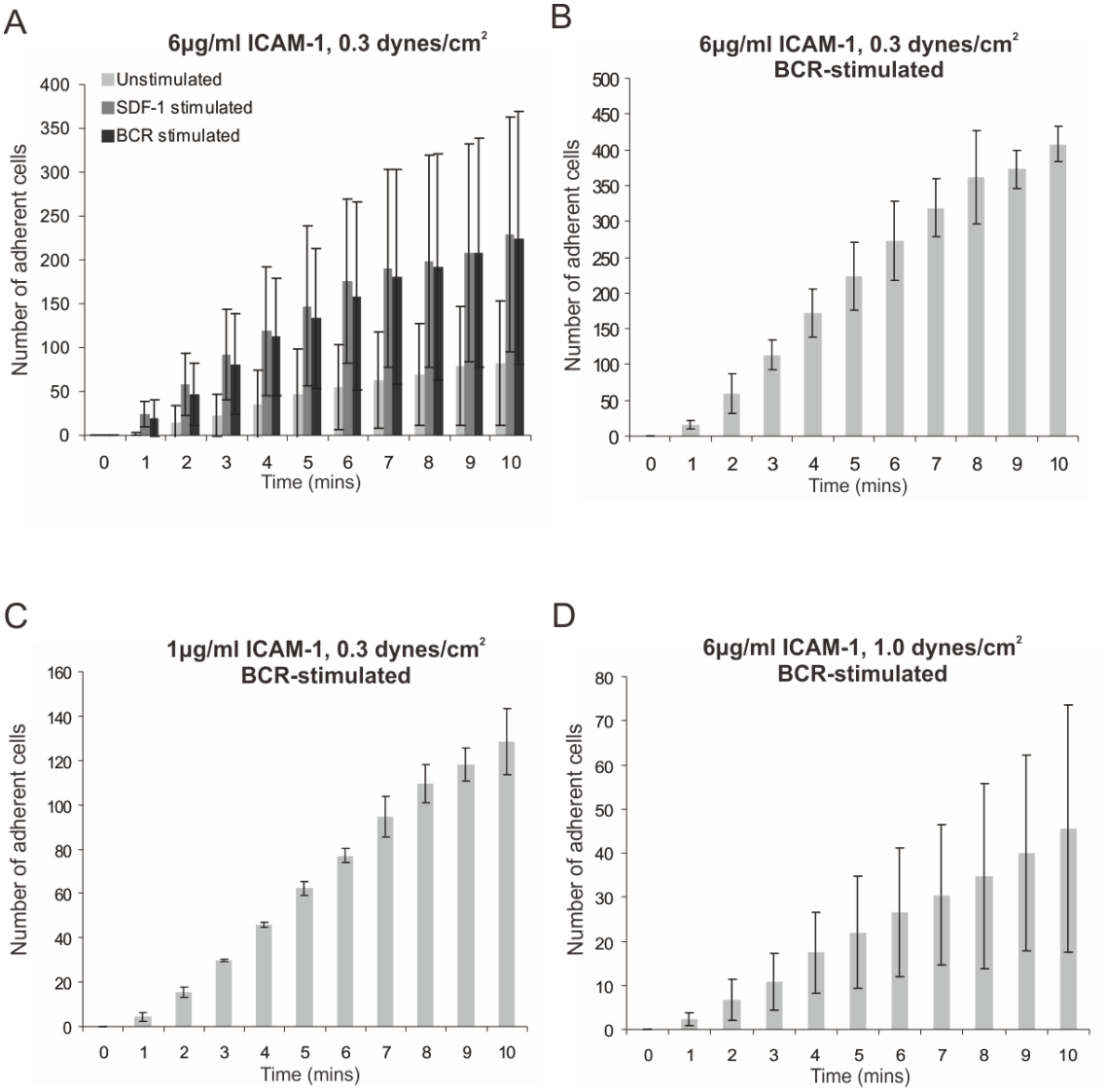


Figure 29. Spleen cells from wild-type mice have low ability to adhere to ICAM-1 and require stimulation by chemokines or through the BCR for adhesion under shear flow conditions.

(A) Adhesion of murine splenocytes on immobilized ICAM-1 under flow conditions at 0.3 dynes/cm^2. Cells were left untreated, stimulated using immobilized SDF-1 or stimulated at the BCR by IgM. to adhere to the immobilized ICAM-1. N=4-6 experiments. p>0.05 (ANOVA) (B-D) Adhesion of BCR-stimulated splenocytes, stimulated with IgM at different ligand (ICAM-1) concentrations, (B) 6 µg/ml, 0.3 dynes/cm^2 (C) 1 µg/ml 0.3 dynes/cm^2 and (D) 6 µg/ml ICAM-1 but at 1 dynes/cm^2 instead of 0.3 dynes/cm^2. N=2 experiments for B-D, with splenocytes from 1 mice used for each experiment. In all graphs error bars represent S.D.

A series of experiments were carried out at low shear stress to determine the best conditions for shear flow experiments. (Data not shown.) 0.3 dynes/cm^2 was chosen as the shear flow rate as this shear stress consistently enabled a few hundred cells to adhere to ICAM-1 ligands and this cell number was manageable to count manually at one minute intervals. Splenocytes that were either stimulated through the B cell receptor or stimulated by adherent SDF-1 chemokine on ICAM-1 adhere much better than unstimulated cells (Figure 29A).

To show that adhesion of splenocytes was dependent on the ligand concentration, shear flow assays using 6 µg/ml ICAM-1 was the recommended concentration used in static adhesion assays (Figure 15). SDF-1 activation of B cells is a more physiologically relevant method to assess cell adhesion in shear flow conditions. However, IgM was used to stimulate the splenocytes at the B cell receptor to assess if activated integrins could resist shear stress in these shear flow assays. Indeed, there were many more cells, approximately 400 adherent cells per field of view using the higher ligand concentration, compared to approximately 130 adherent cells using the lower ligand concentration at the end of the assay (Figure 29B and 29C). When the shear stress was increased from 0.3 dynes/cm^2 to 1 dyne/cm^2, cell adhesion dropped from approximately 400 cells to only 45 adherent cells at the end of the assay (Figure 29B and 29D). Taken together, chemokine stimulation or B cell receptor stimulation is required in shear flow experiments using splenocytes or B cells (depending in the purpose of assay) and the assay conditions for splenocytes and B cells was standardised to 6 µg/ml ICAM-1 and a minimum of 0.3 dynes/cm^2, which were used in subsequent assays unless otherwise stated.

3.3.10. Roles of AGC kinase in murine B cells in shear flow conditions

3.3.10.1. PKC may be important for SDF-1-induced shear flow adhesion.

After the establishment of optimal conditions for B cell shear flow assays, wild-type murine B cells pre-treated with inhibitors or purified from knock-in/knock-out mice were used in these assays. The static adhesion assays in the previous section had shown that PKC isoforms may be required for phorbol ester and BCR-induced static B cell adhesion to ICAM-1, whereas PKD was not necessary for integrin-mediated cell adhesion in these cells under these conditions (Figure 19-21). However, it was unknown whether these pathways were important in chemokine-induced adhesion under conditions of shear flow.

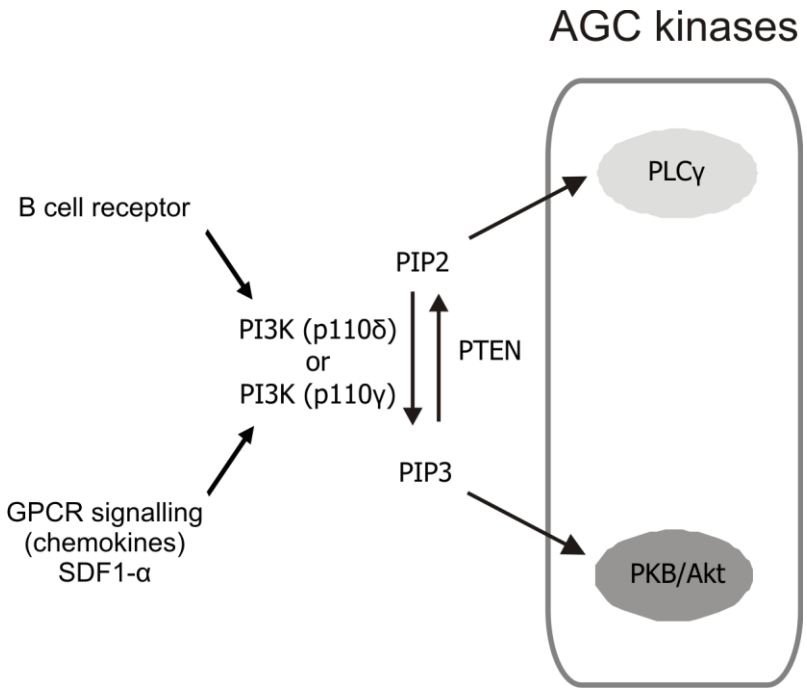


Figure 30. Activation of AGC kinases via B cell receptor signalling or G-protein coupled receptor in B cells.

When cells receive stimulation via chemokines (such as SDF-1α), Class 1B PI3K (p110γ) is activated, leading to the production of membrane bound PIP3. Cells stimulated at the BCR instead activate Class 1A PI3K (p110δ). PIP2 production leads to the activation of PLCγ, which promotes production of DAG and activation of PKC downstream, while calcium is released at the same time. PIP3 is needed in co-activation of PKB/AKT with PI3K.

We therefore assessed the role of PKC in regulating SDF-1-induced B cell adhesion to ICAM-1 under shear flow condition. PKC-inhibitor-treated splenocytes adhered less than untreated cells under shear flow, indicating that PKC may regulate B cell adhesion to some degree also under these conditions (Figure 31).

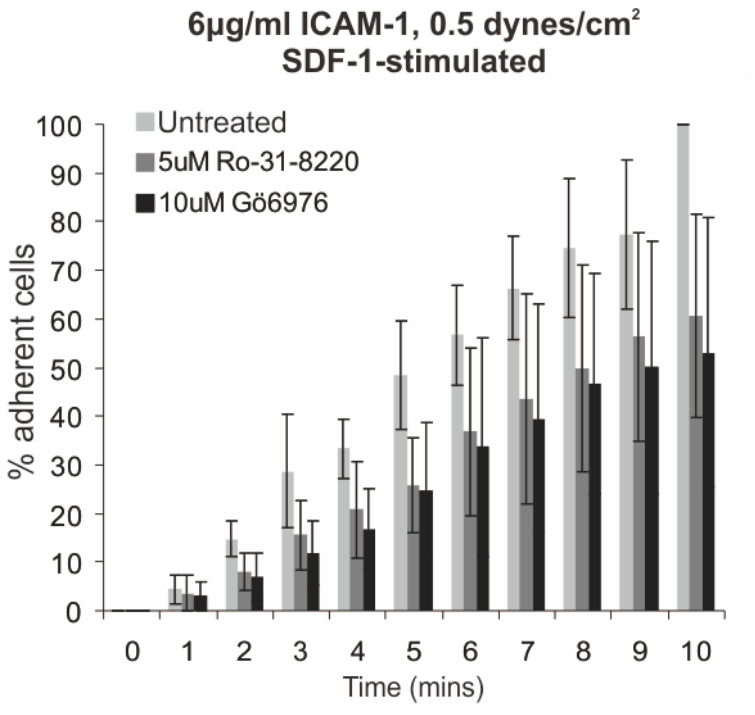


Figure 31. Splenocytes pre-treated with PKC inhibitors displayed reduced SDF-1-induced adhesion under shear flow.

Splenocytes which were untreated or pre-treated with PKC inhibitors 5 µM Ro-31-8220 or 10 µM Gö6976 were allowed to adhere to ICAM-1 overlaid with SDF-1 under shear flow of 0.5

dynes/cm^2. Results are expressed as a percentage when compared to total number of stimulated cells without drug treatment adhered to ICAM-1 at the 10 minute time-point (due to large differences in cell adhesion between different splenocyte batches). N=5 experiments, with splenocytes from 1 mice used for each experiment. p<0.05 (ANOVA). Error bars represent S.D.

3.3.10.2. Inhibiting PI3K/Akt in B cells reduced SDF-1-induced adhesion to ICAM-1 under shear flow.

Results shown in Figure 24 indicated that pre-treatment of resting and B cell receptor stimulated B cells with the PI3K inhibitors, LY294002 and GDC-0941 reduced cell adhesion to ICAM-1 under static conditions. We therefore investigated whether the PI3K pathway was also involved in regulating SDF-1-induced shear flow adhesion in these cells. Shear flow experiments indeed showed that when PI3 Kinase was inhibited using GDC-0941, this affected SDF-1-induced integrin-mediated B cell adhesion to ICAM-1 (Figure 32A). It was decided to just perform this experiment with GDC-0941 as it this inhibitor is more specific than LY294002 as discussed previously (Bain et al., 2007). In addition, static adhesion assays carried out earlier had shown that GDC-0941 can inhibit downstream Akt activity and inhibit integrin-mediated cell adhesion in static conditions (Figure 24).

Static adhesion assays had shown that resting and BCR-stimulated B cells pre-treated with Akt inhibitors (Akt VIII inhibitor or MK2206) adhered less than control cells. In shear flow assays where cells were BCR-stimulated, similar results were observed (Figure 32B and 32C). This strengthened the suggestion that Akt may play an important role in the regulation of integrin-mediated cell adhesion in BCR-stimulated cells.

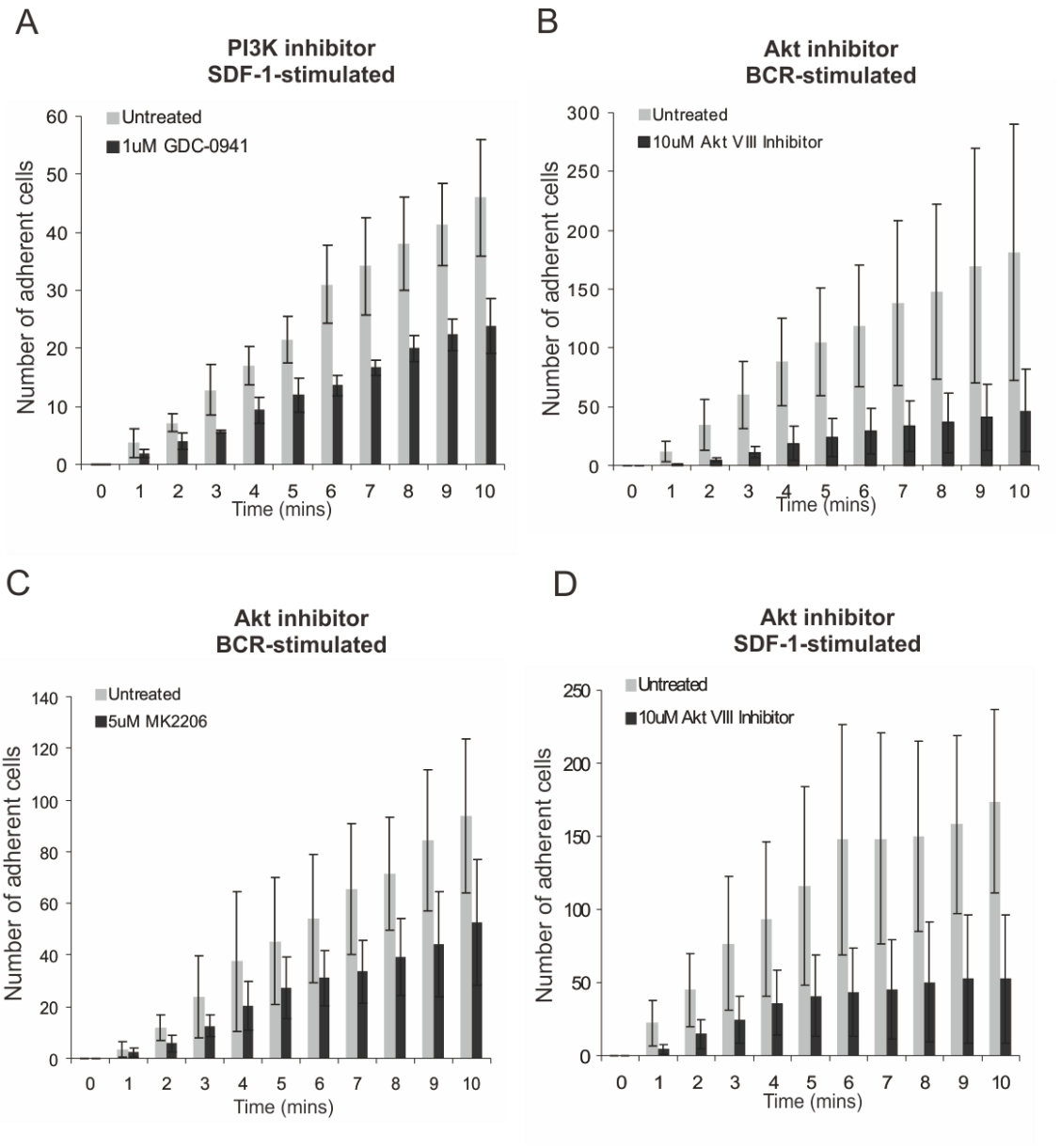


Figure 32. B cells require PI3K/Akt for adhesion to ICAM-1 under shear flow conditions.

(A) Wild-type mice splenocytes, untreated or pre-treated with PI3K inhibitor GDC-0941 and were allowed to adhere to immobolized ICAM-1, stimulated by immobilized SDF-1 uunder shear flow. N=4, P<0.05 (ANOVA.) (B) Adhesion of untreated and Akt VIII inhibitor-treated (10μM) BCR-triggered B cells to ICAM-1 under shear flow conditions. N = 5 experiments. p<0.05 (ANOVA). (C) Untreated or MK2206-treated (5 μM) BCR-triggered B cells were analyzed for adhesion to ICAM-1 under shear flow conditions. N =5 experiments, p<0.05 (ANOVA) after being subjected to shear stress from 4 minutes onwards. (D) SDF-1-induced

adhesion of untreated and Akt VIII inhibitor-treated (10 μM) B cells to ICAM-1 under shear flow. N = 4 experiments, p<0.05 (ANOVA). Shear flow was at 0.3 dynes/cm^2 for all experiments For each experiment splenocytes from 1 mice each was used. In all graphs error bars represent S.D.

Importantly, when B cells were stimulated by immobilized SDF-1 under shear stress conditions, the inhibition of Akt significantly affected cell adhesion to immobilized ICAM-1, indicating that chemokine-induced B adhesion is also regulated by the PI3K/Akt pathway (Figure 32D). This suggested that Akt in B cells is important for cell extravasation out of blood vessels during homing and trafficking to sites of inflammation. In the presence of shear stress, the difference in adhesion properties between Akt VIII inhibitor-treated B cells and control cells was more prominent than that in a static adhesion assay. Cells that adhered bind strongly and were difficult to wash off at higher shear stress. (Data not shown.)

3.3.10.3. Glucose deprivation did not affect cell adhesion under shear flow.

Akt is important in the regulation of glucose metabolism in lymphocytes (Frauwirth and Thompson, 2004). GSK3 (Glycogen Syntase Kinase) which is downstream of Akt is important in glucose transporter GLUT1 regulation which was shown to be important in β1 and β3 integrin trafficking in fibroblasts (Roberts et al., 2004, Wieman et al., 2007). The RPMI-1640 medium used for adhesion assays and shear flow assays contained 2000mg/L glucose, together with other components that promote cell growth and survival (according to manufacturer's product sheet, GIBCO). In order to exclude that

Akt inhibition could affect glucose metabolism which could in turn affect integrin activation and cell adhesion to ICAM-1, we compared shear flow adhesion in in glucose-free adhesion buffer in (PBS) with that in RPMI-1640 medium.

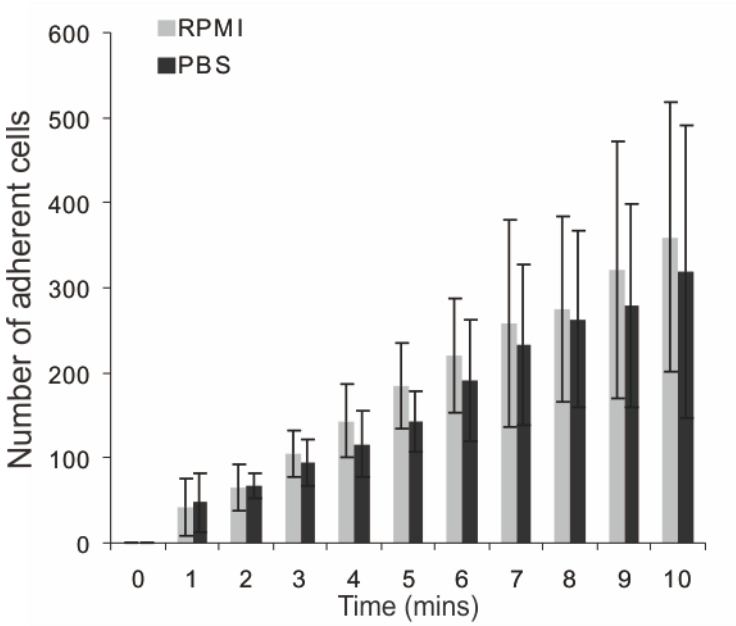


Figure 33. Adhesion of splenocytes in glucose-free medium was similar to glucose-containing RPMI used in adhesion assays.

Glucose deprivation in splenocytes was assessed using glucose-free PBS as adhesion medium, compared to the usual RPMI-based adhesion medium. Adhesion of splenocytes on ICAM-1 under shear flow of 0.3 dynes/cm.^{2} was assessed. N=2 experiments (1 mouse per experiment), p>0.05 (ANOVA) Error bars represent S.D.

The result shows that there was no significant difference in cell adhesion of splenocytes using the PBS-based adhesion medium e.g. in the absence of glucose versus in the presence of glucose (Figure 33). This result also showed that the PBS-based buffer could also be used for adhesion assays to exclude other components such as amino acids, vitamins or calcium if necessary.

3.3.11. SDF-1-induced T cell adhesion under shear flow conditions was unaffected by Akt inhibition.

The SDF-1 chemokine that was used to stimulate B cells in our assays can also be used to stimulate T cell adhesion (Schreibe et al., 2007). Shear flow assays were therefore performed to investigate whether SDF-1-induced T cell adhesion under shear flow was dependent on Akt.

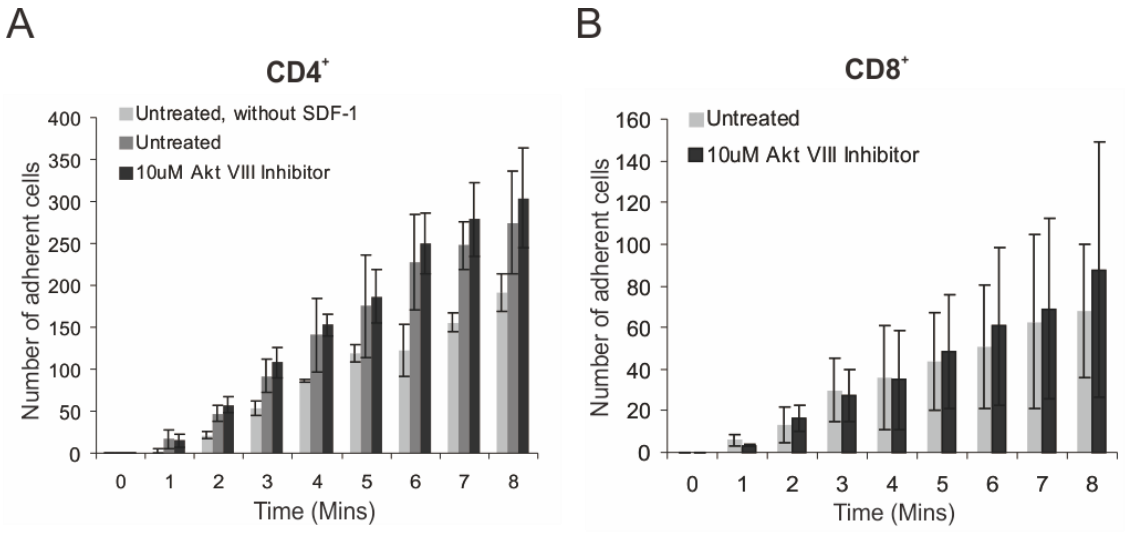


Figure 34. CD4$^+$ and CD8$^+$ T cells do not require Akt for adhesion to ICAM-1 under shear flow conditions.

(A) SDF-1 induced adhesion of CD4$^+$ T cells to ICAM-1 under shear flow of 0.5 dynes/cm^2 using untreated or Akt VIII inhibitor-treated (10 μM) cells, N=2 experiments, p>0.05 (ANOVA) for SDF-1 treated samples. Error bars represent S.D. (B) SDF-1 induced adhesion of CD8$^+$ T cells to ICAM-1 under shear flow of 0.3 dynes/cm^2 using untreated or Akt VIII inhibitor-treated (10 μM) cells, p>0.05 (ANOVA). Error bars represent S.D. N=2 experiments. (Purified cells from 1 mice each was used per experiment.)

Unstimulated $CD4^+$ T cells adhered to ICAM-1 under shear flow conditions. As $CD4^+$ T cells tended to be "stickier" than B cells at 0.3 dynes/cm^2, the shear rate was increased to 0.5 dynes/cm^2 to avoid having too many cells to count per minute leading to errors. (Data not shown.) Even at this higher shear rate, many unstimulated $CD4^+$ T cells adhered to ICAM-1, as there was approximately 200 adherent $CD4^+$ T cells at 0.5 dynes/cm^2 at the end of the assay compared to approximately 50 adherent splenocytes cells at 0.3 dynes/cm^2 (Figure 29A and Figure 34A). Stimulating $CD4^+$ T cells with SDF-1 slightly increased adhesion of T cells to ICAM-1 but pre-treatment of cells with the Akt VIII inhibitor did not affect cell adhesion to ICAM-1 (Figure 33A). SDF-1 stimulated $CD8^+$ T cells were less adherent than $CD4^+$ T cells to ICAM-1 so the assay was carried out at 0.3 dynes/cm^2. Treatment of $CD8^+$ T cells with the Akt VIII inhibitor did not significantly decrease their adhesion (Figure 34B). These results suggested that Akt does not play a role in T cell adhesion under flow conditions, and inhibition of Akt in $CD4^+$ or $CD8^+$ T cells would not affect extravasation out of blood vessels, unlike the situation in B cells, where Akt does appear to play a role in shear flow adhesion (Figure 32 and Figure 34). Therefore, cell adhesion under shear stress conditions appears to be differently regulated in B cells and T cells.

3.4. Discussion

In this chapter, we examined the role of some AGC kinases in the regulation of integrins in B cells in different types of B cell adhesion. Static cell adhesion assays, where B cells are stimulated by BCR-ligation, mimic B cell activation by antigen-presenting cells. The chemokine SDF-1α in turn was used to stimulate lymphocytes in shear flow assays. This assay mimics the situation in a blood vessel where these

chemokines are found on the endothelial cell layer, and stimulate lymphocytes through their G-protein coupled receptors during cell rolling, which leads to firm adhesion of the lymphocyte to the endothelial cells. SDF-1α also provides a gradient in tissues which enable lymphocytes to migrate to sites of injury after extravasation from blood vessels. Therefore different types of adhesion assays and cell stimulation used in the absence or presence of shear flow were necessary for mimicking different in vivo conditions.

The static adhesion assays results were expressed as fold changes due to variability in cell adhesion between B cells isolated from different mice. For each experiment, cells from 2-3 mice were pooled together to get enough B cells. However, it was soon noticed that percentage of total, unstimulated, non-inhibitor treated control cells adhering to ligands could vary greatly between experiments carried out on different days even when the age of wild-type mice was kept to 6-8weeks. Although, for many of the experiments, the adhesion percentage recorded for control cells adhering to ligands were between 20-30%, sometimes adhesion was nearly 90%. This variance between lots of cells would make it difficult to compare cell adhesion percentage between treatments by simply comparing the average of "percentage of cells adhered" between treatment groups. Therefore, fold-changes comparing different treatment groups to the unstimulated, non-inhibitor treated cells were used to display the results from these assays.

It was also noted that in static adhesion assays using immobilized fibronectin, stimulation of B cells/splenocytes by phorbol ester or at the B cell receptor using IgM generally did not induce a significant increase in cell adhesion, indicating that fibronectin-binding integrins may not be significantly activated by phorbol ester or BCR-stimulation in primary B cells. For static adhesion assays using immobilised

ICAM-1, phorbol ester stimulation could stimulate LFA-1-mediated cell adhesion to ICAM-1 in most experiments while simulation of cells at the B cell receptor had a smaller effect. However, BCR-induced adhesion to ICAM-1 could be detected under flow conditions, indicating that this assay may be better than a static adhesion assay for investigating BCR-stimulated adhesion to integrin ligands.

We found that PKC isoforms may be important for integrin-mediated BCR-induced, phorbol ester-induced and possibly SDF-1-induced B cell adhesion, but PKCβ, the most abundant PKC isoform in B cells did not affect LFA-1 or fibronectin-binding integrin activation. The reason for this may be that the presence of ten other PKC isoforms could perhaps compensate for the loss of function of PKCβ in integrin activation (Tan and Parker 2003, Parker 2004, Kong et al., 2013). It is difficult to find out which PKC isoforms are important in integrin regulation in B cells as there are no suitable inhibitors which are able to inhibit individual PKC isoforms. However, the use of inhibitors enabled us to narrow down subgroups of PKCs which may be important for B cell adhesion. PKC knock-out mice of individual isotypes had been made and used in a few laboratories such as Michael Leitges, who provided the PKCβ knock-out mice spleens used in this study; some of these may be used in the future to investigate the role of individual PKC isoforms in regulation of B cell adhesion. Some of the possible knock-out mice include the PKCδ (Leitges et al., 2001), PKCζ (Leitges et al., 2001) or the much researched PKCθ (Pfeifhofer et al., 2003). However, it is possible that the effect of deleting one PKC isoform may be subtle, leading to an only mild decrease in cell adhesion to ICAM-1 due to compensatory mechanisms.

As mentioned by Cohen, 2000, a selective and suitable kinase inhibitor should block a biological process at the same concentration of blocking a kinase activity. However, the

use of PKC inhibitor Gö6976 reduced cell adhesion at 0.5µM but did not reduce phospho-PKC (PKD) levels at 1µM. This indicated that Gö6976 could inhibit other kinase aside from PKC which had an effect on cell adhesion. Whether if Akt or other kinase are affected by this inhibitor should be investigated and screened for in future.

We have shown here that PKD, especially the most abundant isoform in lymphocytes, PKD2 (which is a direct downstream effector of PKC) is not required for integrin mediated adhesion. PKD is also not required for regulation of Rap1 in lymphocytes. This is different to what had been shown by Medeiros et. al. in 2005. This could be due to difference using cell line model instead of primary cells and also differences between B and T cell types. Work carried out by Sharon Matthews had also shown that lymphocytes expressed high levels of PKD2 and some PKD3 but not PKD1 (Matthews et al., 2010, Matthews et al., 2012). As the previous group studied PKD1 while our work focused on the most abundant isotype PKD2, Novartis 12a inhibitor that had been shown to be able to inhibit all 3 isoforms of PKD was used in adhesion assays (Matthews et al., 2012). The results showed that the lack of any PKD did not affect integrin-mediated cell adhesion. In addition, we used in vivo migration studies to investigate PKD2 knock-out lymphocyte adhesion and homing to tissues. Lymphocytes isolated from WT and PKD2-deficient mice were labelled with carboxyfluorescein succinimidyl ester (CFSE) or CellTrace Violet/CMTMR dyes (Molecular Probes, Invitrogen), respectively; washed and mixed in a 1:1 ratio in sterile PBS. Cell suspensions (10^7cells) were then injected into the tail vein of C57BL/6 host mice, and 3 hours later the mice were killed and tissues taken for quantification of CFSE and CellTrace Violet-labelled T and B cells analysed by flow cytometry (Matthews et al., 2012). The loss of PKD2 did not affect lymphocyte homing to lymphoid organs. This

result further shows that PKD2 is not responsible for regulating integrin activation in lymphocytes.

What is the link between Akt and integrins? From our results it appears that Akt may influence Rap1 activation of integrins through an unknown mechanism. The A20 cell line was used for Rap1 assay instead of primary B cells as A20 had high levels of active Rap1, and because of the limitations of cell numbers when working with primary B cells. Akt has been found to be overexpressed or dysregulated in cancer cell lines (Hyun et al., 2000, Chen et al., 2010). Pre-treatment of A20 cells with the Akt VIII inhibitor led to reduced active Rap1 in resting and B cell receptor triggered cells. It is currently not certain if the same is true in murine primary B cells, because it was difficult to obtain enough primary B cells to perform this assay in these cells. One possibility would have been to use splenocytes for these assays, but Akt VIII inhibitor worked more effectively in reducing B cell adhesion and lowering phospho-Akt (308) levels in purified B cells than in splenocytes. (Data not shown.) Nonetheless, there is still enough evidence to suggest the existence of a novel BCR-induced signalling pathway involving Akt in the regulation of Rap1, which in turn activates integrins. It may also be that a similar signalling pathway is at work in shear flow conditions when B cells are stimulated by chemokines before the extravasation process. Interestingly, a link between Akt and Rap1 has recently been suggested in patients with myeloproliferative disorders. These patients had a mutation in the JAK kinase 2, and were found to have reduced levels of phosphorylated Akt and also reduced activation of Rap1 which affected $\alpha_{IIb}\beta_3$ integrin activation in platelets (Moore et al., 2013).

How might Akt influence Rap1 regulation in B cells? Rap1 regulation in cells is favoured by activating Guanine nucleotide exchange factors (GEFs) such as C3G,

CalDEG-GEF, Epac, DOCK-4 (Ghandor et al., 2007, Pannekoek et al., 2008) or inhibition of Rap1 inhibiting GTPase activating proteins (GAPs) such as Rap1GAP, Rap1GAPII, Spa-1(SIPA1) and SIPA1L1 (Katagiri et al., 2002, Letschka et al., 2008, Tsygankova et al., 2010). Mathematical modelling performed by our collaborator Daniela Schlüter in the department of Mathematics, University of Dundee, showed that it was highly possible that modulation of Rap1 by Akt in B cells was regulated by GAPs rather than GEFs. (Data not shown.) Indeed, Rap1GAP has been shown to be important in regulation of Rap1 in HUVECS (human umbilical vein endothelial cells), where overexpression of Rap1GAP reduced Rap1 levels and affected cell proliferation (Li et al., 2011) (Figure 35). Rap1 has previously been suggested to affect Akt and ERK phosphorylation (Wang et al., 2001, Li et al., 2011). However, in our case, Akt could regulate Rap1 instead.

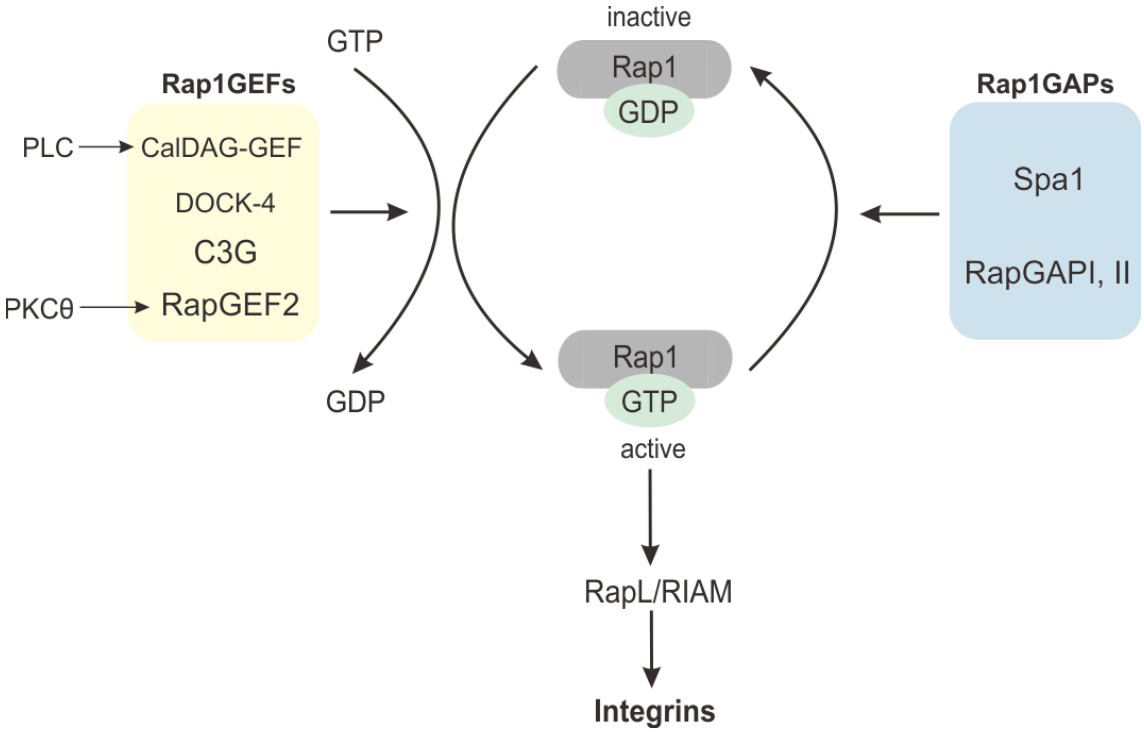


Figure 35: Regulation of Rap1 by GEFs and GAPs

Resting Rap1 is GDP-bound but can be activated when GTP-bound, leading to the regulation of RapL/RIAM which activate integrins. Activation of Rap1GEFs such as CalDAG-GEF (activated by PLC), DOCK-4, C3G and RapGEF2 (activated by PKCθ) release GDP from Rap-1 and thereby generate more GTP-bound Rap1.Rap1GAPS such as Spa1, RapGAPI and RapGAPII inhibit Rap1 activation by aiding GTP hydrolysis to GDP. Akt may regulate Rap1 by suppressing GAPs, as discussed in the text.

Interestingly, the Rap1GAP Spa-1 (Jin et al, 2006) has been found to be phosphorylated on a putative Akt-phosphorylation site (Ser72) in effector cytotoxic T cells, and is therefore a possible link between Akt and Rap1 in lymphocytes (Navarro et al., 2011). Whether this site is phosphorylated in B cells is unknown at present, as antibodies to detect phosphorylated Spa-1 are not currently commercially available. Spa-1 overexpression in HeLa cells caused cells to detach and round up, indicating that Spa-1 may regulate integrin-mediated adhesion (Tsukamoto et al., 1999). In the future, to investigate if phosphorylation of Spa-1 by Akt regulates Rap1 activity in cells, cells (such as COS-cells) could be transfected with Spa-1 or Ser72Ala-mutated Spa-1 (so that the site cannot be phosphorylated due to the lack of –OH group in Alanine). These cells could then be pre-treated with Akt inhibitors and the effect the mutation and of Akt inhibition on Rap1 activity after cell stimulation could be assessed using the Rap1 pulldown assay. If Spa-1 is phosphorylated by Akt to regulate Rap1 activity, Akt inhibitor treatment of control Spa-1/Rap1 cells would yield lower Rap1 levels than without Akt treatment of such cells. Stimulated S72A Spa-1/Rap1 transfected cells, with or without Akt inhibitor treatment, would have similar levels of Rap1 activation.

PKD1 was also suggested to be necessary for integrin-mediated adhesion via Rap1 regulation (Medeiros et al., 2005). Our work has shown that lack of PKD1 or PKD2, using Novartis 12a PKD inhibitors or PKD2 knock-out mice splenocytes did not affect

Rap1 activity. Rap1 is also a downstream effector of PKC in T cells (Letschka et al., 2008). Resting B cells or B cells stimulated with phorbol ester had significantly decreased active Rap1 levels when pre-treated with Ro-31-8220 or Gö6976, indicating that Rap1 may be the downstream target of PKC in integrin regulation under these conditions. In contrast, B cells pre-treated with these PKC inhibitors but stimulated through the B cell receptor only showed a slight decrease in active Rap1 levels but adhesion was still reduced (Figure 27). Therefore, under these conditions, PKC may regulate integrins through a Rap1-independent mechanism.

Such a Rap1-independent mechanism has indeed been previously described in T cells by Ghandour et al. (2007), where CD3+ T cells can either bind to ICAM-1 via Rap1 activation of LFA-1 or to VCAM-1 via VLA-4 without Rap1 activation. Silencing Rap1 guanine exchange factor CalDAG-GEFI reduced LFA-1 mediated cell adhesion to ICAM-1 (when cells were stimulated by SDF-1 or by PMA) but adhesion to VCAM-1 was unaffected. Inhibition of PLC reduced adhesion to both ICAM-1 and VCAM-1 while inhibition of the downstream component PKC reduced adhesion of SDF-1α stimulated T cells to VCAM-1 but increased adhesion to ICAM-1. The authors' conclusion was that integrins on T cells can be differentially regulated by PMA or chemokine stimulation. PLC/CalDAG-GEFI regulates Rap1 which leads to activation of LFA-1, whilst chemokines can also activate an alternate Rap1-independent signalling pathway where PKC activates VLA-4 (Ghandour et al., 2007). However, it is still largely unknown how PKC activates VLA-4 integrins after chemokine stimulation.

Rap-1-mediated LFA-1 activation has also previously been shown to be regulated through a PKCθ/ RapGEF2 pathway when stimulated at T cell receptor (Letschka et al., 2008). In addition, primary $CD3^+$ T cells expression of SPA-1 or Rap1GAP inhibits

LFA-1 activation (Ghandour et al., 2007). We suggest that in B cells, Rap1 is regulated through Akt-mediated inhibition of Rap1Gap or Spa1, leading to LFA-1 activation and ICAM-1 binding when stimulated at the B cell receptor in static conditions (Figure 35). However, further investigations are needed to clarify these issues and if Akt regulation of Rap1 is different when B cells are stimulated by chemokines in static and shear flow conditions.

In this chapter, shear flow assays were successfully developed for B cells and $CD4^+$, $CD8^+$ T cells. Although the shear stress was slightly lower than in physiological conditions, cells stimulated by chemokines could be activated and stimulated to adhere to ICAM-1 under shear flow conditions. It was also observed that although adhesion of lymphocytes could occur at higher shear stresses of 30 dynes/cm^2 in vivo (Morigi et al., 1995), adhesion of lymphocytes was observed to occur up to 5 dynes/cm^2 in vitro. Reasons for this were not established. Integrins in B and T cells were found to be differentially regulated by Akt, as Akt was not required in T cells for integrin-mediated adhesion under shear flow conditions.

Based on the results from these studies, it was hypothesized that Akt inhibition would affect B cell migration and homing in vivo. In vivo experiments with untreated and Akt inhibitor-treated B cells to assess B cell homing were carried out (not shown), but there was no observable difference in B cell homing to lymphoid organs between these conditions. This could be due to a “wash-out” effect of the Akt inhibitor from the cells during the in vivo experiment, which lasted several hours. (Data not shown. Experiments similar to that using PKD2 knockouts discussed earlier and carried out together with Vicky Morrison.) Indeed, Western blot detection of phosphorylated Akt (308) levels demonstrated that the effect of the Akt inhibitors on Akt phosphorylation

started to wear off and after 2 hours the phosphorylation level reached control levels. (Data not shown.)

Although the use of inhibitors had been debated as an unreliable way of studying some kinases due to non-specificity and lack of kinase screening data available (Bain et al., 2007), such experiments are useful as an initial process to test the hypothesis. An alternative and better method to confirm the involvement of a protein kinase regulating integrin function is to use transgenic mice for such studies. There are 3 isoforms of Akt expressed in mammalian cells. Akt1 is expressed in all cells and is required for growth and development and has pro-survival signalling properties while Akt2 is important in insulin-responsive cells. Akt3 is expressed at low levels in all tissues but is highly expressed in the brain and testis (Chen et al., 2010).

For in vivo studies, other researchers have generated knock-out mice of various Akt isoforms and combinations of these but it is not possible to generate viable mutant mice lacking all 3 isoforms or lacking Akt1. Akt1, which is of most interest to our studies, is necessary for embryonic development and postnatal survival (Dummler et al., 2006). One option is to use Cre/loxP system to generate Akt1 ($^{flox/flox}$) mice which are tamoxifen-inducible for transient knock-out of Akt1; these could be used to study the role of Akt in B cell adhesion in vitro (Feil et al., 2009). These cells could also be mixed with equal population of wild-type B cells, injected into mice to follow B cell homing in vivo as discussed earlier to replace Akt inhibitor treatment. Another possible method to study Akt would be to obtain mice that overexpress Akt in lymphocytes to assess if this has any significant effect on lymphocyte adhesion. These mice express myristoylated Akt1 (Na et al., 2003). It has been reported that B cells with enhanced Akt1 expression had increased migration towards SDF-1 chemokine but displayed less BCR–stimulated

proliferation and signalling. Adhesion to ICAM-1 in static conditions did not significantly increase but adhesion to fibronectin was slightly increased in B cells with enhanced Akt1 expression when stimulated with SDF-1α (Bommhardt et al., 2004, Pierau et al., 2012), suggesting that Akt regulation is needed for migration to sites of infection via fobronectin-binding integrins. Importantly, these experiments were conducted in static conditions and could be performed with the presence of shear flow to investigate if enhanced Akt expression affects adhesion to different integrin ligands (such as ICAM-1 and VCAM-1) and migration when stimulated with SDF-1α.

Tamoxifen-inducible knock-out of PDK1, which is upstream of Akt and thought to regulate many AGC kinases, is available and has been reported to have ablated phosphorylated of Akt at Thr308 in cardiac muscles and this was also observed in T cells of similar knock-out mice (Mora et al., 2003, Macintyre et al., 2011). Therefore it may be possible to use PDK1 knock-out cells to investigate the role of Akt in lymphocyte integrin regulation in vivo. We therefore attempted to perform shear flow adhesion assays with PDK1 knock-out B cells. Unfortunately, cell numbers were limited using this approach and the results were inconclusive. (Data not shown.) Many AGC kinases are targets for cancer drug development as they are implicated in controlling apoptosis and migration of cancerous cells (Sato et al., 2002). Investigating the role of PDK1/AGC kinases in signalling pathways in lymphocyte adhesion and migration processes will improve the understanding of the role of these kinases in lymphocyte biology and in disease conditions.

Lastly, it is not currently clear how AGC kinases Akt and PKC interact with each other. There are suggestions that PKC negatively regulates Akt (Li et al., 2006) and novel PKC inhibitors like Ro-31-8220 activate Akt (Wen ei al., 2002, Liu et al., 2006).

However, some other earlier reports suggested that overexpression of PKCα also stimulates Akt, (Li et al., 1999) but these investigations have not been conducted in lymphocytes. At this stage, the use of inhibitors only was not enough to conclude of the relationship between PKC and Akt in integrin regulation. Adhesion assays were conducting using both types of inhibitors to investigate if inhibition of both kinases had an additive effect or able to compensate for the lost of effect from the other kinase. However, the results remain unconclusive. (Data not shown.) Better and consistent results could be obtained using mice lacking PKC or Akt and treating it with the other inhibitor.

3.5. Conclusion

In this chapter, we have shown that PKC isoforms, but not PKCβ or PKD2, may be important in the regulation of integrin activation in B cells. PKC is upstream of Rap1 activation in resting B cells or in phorbol ester stimulated B cells, leading to B cell adhesion to ICAM-1. However, upon stimulation of the B cell receptor, PI3K/Akt signalling is more important in regulating integrin activation and may work through Rap1. These results suggested that AGC kinases such as PKC and Akt may be important in regulating inside-out signalling of integrins resulting in cell adhesion between B cells and antigen presenting cells. Furthermore, we have developed shear stress B cell adhesion assays to mimic the adhesion process in blood vessels. B cell adhesion under these conditions required SDF-1α chemokine activation to initiate integrin inside-out signalling, resulting in cell adhesion. These studies supported the observations that PKC and PI3K/Akt signalling pathways were involved in integrin activation in B cells. These results have shown that although some PKCs may have a

role in B cell integrin regulation, SDF-1α leads to the inside-out signalling of integrins via PI3K/Akt, leading to firm adhesion of lymphocytes under shear flow before transmigration out of blood vessels into tissues. We have also shown that adhesion of B and T lymphocytes are differentially regulated upon stimulation: Although B cells required PI3K/Akt signalling to regulate integrin-mediated cell adhesion; T cells do not depend on Akt for cell adhesion. Further studies are required to elucidate the mechanism by which Akt regulates B cell adhesion and the role of Akt in integrin regulation in B cells in vivo.

4. Regulation of integrin-mediated adhesion in primary murine effector cytotoxic T lymphocytes

4.1. Introduction

4.1.1. Effector cytotoxic T lymphocytes in the adaptive immune system

In the adaptive immune system, circulating $CD4^+$ or $CD8^+$ T cells are activated in lymphoid organs when they meet a dendritic cell that presents the antigen for T cell priming (Balkow et al., 2010). In order for T cells to be activated, integrin-mediated cell-cell contact is necessary (DeNucci et al., 2009). During inflammation, ICAM-1 is upregulated on various cell types, including T cells. (Arkin et al., 1991). This close contact is mediated by LFA-1-ICAM-1 interactions at the immunological synapse to facilitate the interactions of the T cell with the antigen presenting cell. The contact can last for several hours (Peters et al., 2012). These activated T cells then differentiate to effector T cells, either cytotoxic ($CD8^+$) T cells which kill infected cells or helper ($CD4^+$) T cells that help to activate macrophages or activate B cells to produce antibodies (Broere 2011, Peters et al., 2012). Effector cytotoxic T lymphocytes, like B cells, express high levels of β2 integrins, mainly LFA-1. Integrin-mediated cell adhesion is required for effector functions of T cells. For example, in effector cytotoxic T lymphocytes LFA-1-ICAM-1 interactions facilitate antigen-mediated cell killing, through positioning of granules near the interface of the T cell and target cell, which aids efficient degranulation and cell lysis (Anikeeva et al., 2005).

4.1.2. Effector cytotoxic T lymphocyte migration

Naïve T cells and effector cytotoxic T lymphocytes have different migratory patterns due to the functions they serve. Naïve T cells recirculating via blood and lymphatics express CCR7 which binds to CCL19 and CCL21 in lymphoid tissues. Naïve T cells also have high expression of CD62L/L-selectin which bind to ligands in high endothelial venules, enabling these cells to migrate from blood across high endothelial venules into secondary lymphoid tissues such as lymph nodes. On the other hand, effector cytotoxic T lymphocytes are able to downregulate CCR7 and CD62L/L-selectin and can therefore be redirected away from lymphoid tissues towards non-lymphoid tissues and sites of inflammation (Waugh et al., 2009, DeNucci et al., 2009). At the same time, the tissue homing receptors such as VLA-4 integrins are upregulated to mediate tissue homing (Sinclair et al., 2008). Interestingly, AGC kinases appear important in the control of effector cytotoxic T lymphocyte migration. The strength of Akt activity is important for the control of the migratory program in T cells but not for growth and proliferation (Waugh et al., 2009). Effector cytotoxic T lymphocytes are not able to migrate to non-lymphoid tissue if Akt activity levels are low. It has been shown that effector cytotoxic T lymphocytes pre-treated with Akt inhibitor has increased expression of CD62L and CCR7 which slows down migration. PDK1/Akt activity is important for silencing CD62L/L-selectin expression, which regulates trafficking of T cells. The role of AGC kinases in regulating integrin activation in effector cytotoxic T lymphocytes is unknown at present.

4.1.3. Effector cytotoxic T lymphocyte adhesion under shear flow conditions

Effector cytotoxic T lymphocytes express high levels of LFA-1 (Galkina et al., 2005), and research has shown that LFA-1 were needed by T lymphoblasts to migrate through high endothelial venules and blocking of VCAM-1 using antibodies also reduced adhesion (Faveeuw et al., 2000). Interestingly, however, effector cytotoxic T lymphocytes can bypass chemokine signals and establish firm adhesion to the endothelial layer without chemokine stimulation, but transendothelial migration still required chemokines stored in vesicles beneath the endothelial layers, which bound to G protein coupled receptors on the surface of the effector cytotoxic T lymphocytes to initiate signalling (Shulman et al., 2011).

Effector cytotoxic T lymphocyte integrin regulation is poorly understood. However, it has been reported that Kindlin-3 is important in the regulation of effector cytotoxic T lymphocyte adhesion under shear flow (Manevich-Mendelson et al., 2009). Also, an LFA-1/Lck/Zap70 complex is thought to facilitate rapid integrin-mediated adhesion strengthening in effector cytotoxic T lymphocytes at sites of vascular injury. High levels of phosphorylated Zap70 and talin were observed at the lamellipodia and at the tail end of the cell as the cells migrated under shear flow. Lck and Zap70 are phosphorylated and strengthen the LFA-1-ICAM-1 bond. The lack of Zap70 caused reduced migration across ICAM-1 surfaces and also reduced cell arrest on ICAM-1 (Evans et al., 2011).

4.2. Aims of this Chapter

The aim of this chapter was to investigate how effector cytotoxic T lymphocyte adhesion was regulated. In addition, we wanted to expand shear flow assays to assess effector cytotoxic T lymphocyte adhesion and determine how the presence of shear flow would affect effector cytotoxic T lymphocyte adhesion when compared to cell adhesion in static conditions. Lastly, we wanted to understand how similar or different B cell and effector cytotoxic T lymphocyte integrin-mediated cell adhesion was.

4.3. Results

4.3.1. Integrin expression in effector cytotoxic T lymphocytes

Effector cytotoxic T lymphocytes expressed high levels of β2 integrins and αL integrins.

To examine effector cytotoxic T lymphocytes expression of β2 integrins, flow cytometry to detect surface integrin expression was carried out.

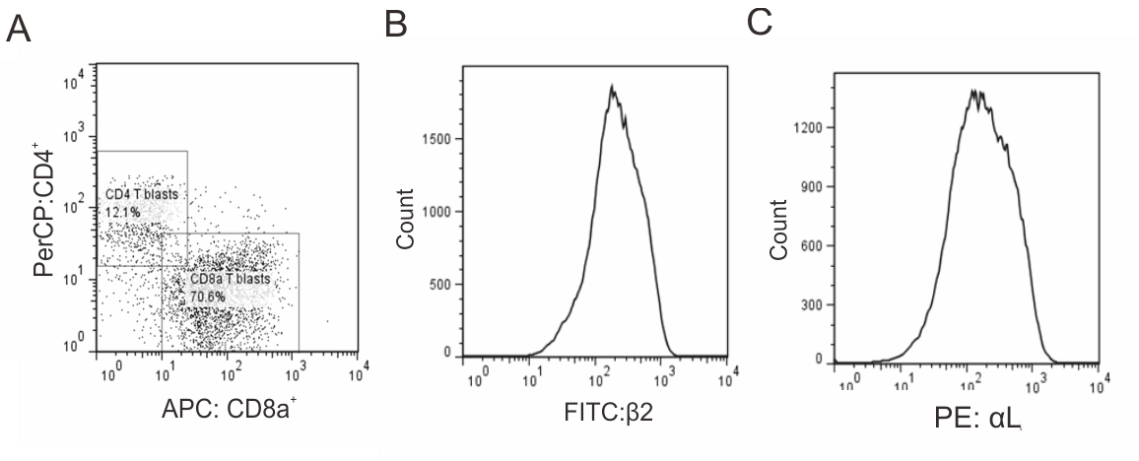


Figure 36. Expression profile of CD18 (β2) and CD11a (αL) integrins in IL-2 maintained effector cytotoxic T lymphocytes.

Effector cytotoxic T lymphocytes (splenocytes stimulated by anti-CD3 for 2days and then grown in IL-2 for the past 7 days) from wild-type mice were stained with $CD4^+$-PerCp Cy5.5, $CD8a^+$-APC, CD18(β2)-FITC and CD11a(αL)-PE antibodies. (A) Dot-plot display of $CD4^+$-PerCp Cy5.5 positive cells (on the y axis) and $CD8a^+$-APC positive cells (on the x-axis). (B) Histograms displaying counts of$CD8a^+$ effector cytotoxicT lymphocytes with detectable levels of CD18 (β2)-FITC and (C) CD11a (αL)-PE fluorescence. Experimental data was obtained from effector cytotoxic T lymphocytes grown from 1 wild-type mouse spleen.

The majority of the effector T cells were $CD8a^+$ positive instead of $CD4^+$ after 7 days in culture (Figure 36A). The effector cytotoxic T lymphocytes express high levels of β2 integrins and αL integrins (Figure 36B and 36C). Comparisons have been made with B cells and populations of $CD4^+$ or $CD8^+$ purified T cells. Effector cytotoxic T lymphocytes generally expressed more β2 integrins than B cells or $CD4^+$ T cells. (Data not shown, experiment performed by Vicky Morrison.)

4.3.2. Effector cytotoxic T lymphocyte adhesion in static conditions

Effector cytotoxic T lymphocytes have the ability to adhere to fibronectin and ICAM-1 ligands.

Cells that express integrins should have the ability to adhere to ligands such as fibronectin via β1 integrins such as VLA-4, VLA-5 and ICAM-1 via αLβ2 integrin, also known as LFA-1. We had shown that effector cells express αLβ2 integrins and the next step was to find out if these cells adhere to ligands and if activation of integrins by stimulation of effector cytotoxic T lymphocytes was needed for cell adhesion.

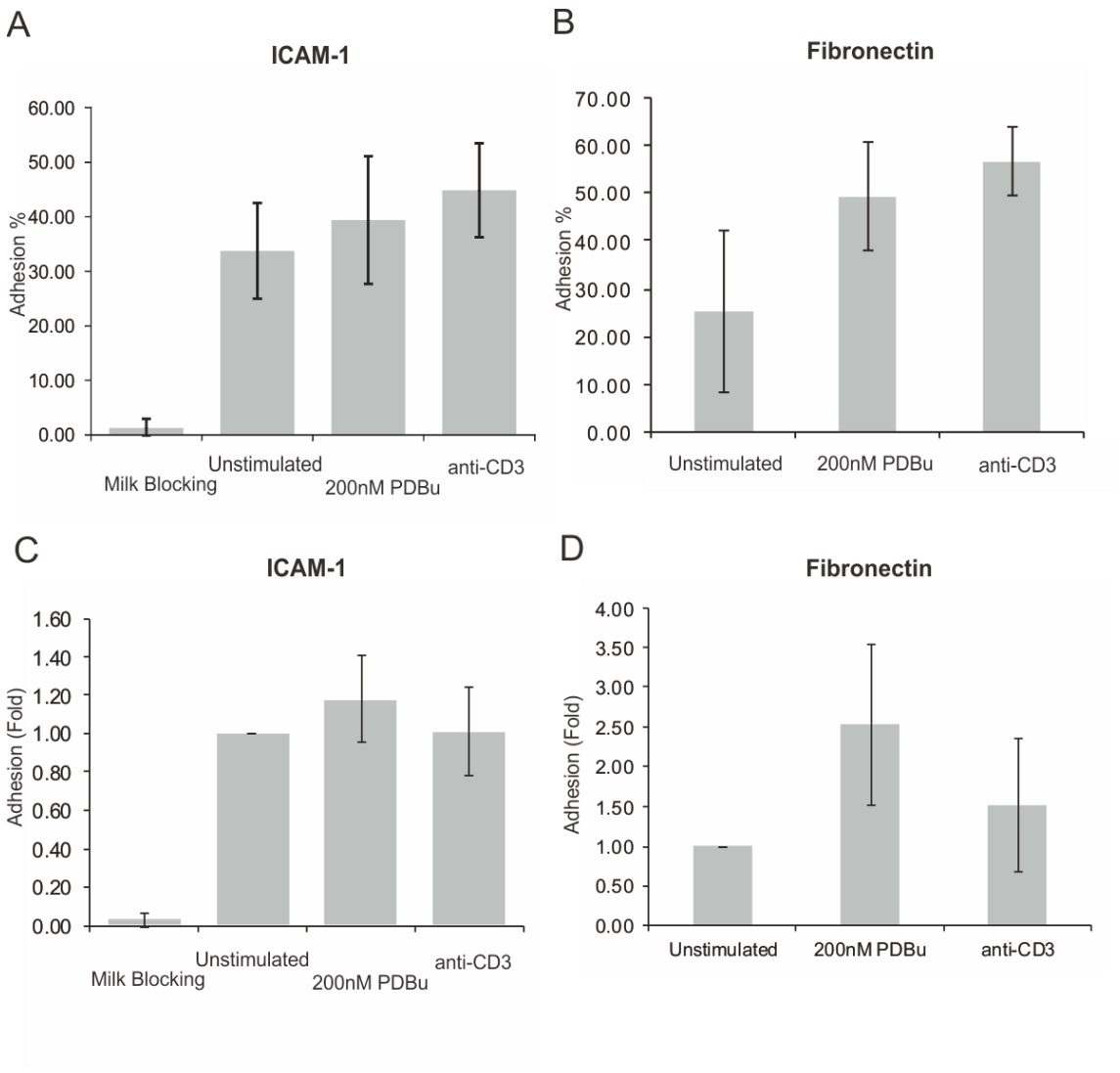


Figure 37. Effector cytotoxic T lymphocyte adhesion to ICAM-1 and fibronectin.

(A) Assessment of unstimulated effector cytotoxic T lymphocytes to a plastic surface with milk coating, or with ICAM-1 coating at 6 µg/ml. Effector cytotoxic T lymphocytes were either unstimulated, stimulated by phorbol ester or with anti-CD3 antibody. (B) Assessment of effector cytotoxic T lymphocytes adhesion to 10µg/ml fibronectin in unstimulated, phorbol ester or anti-CD3 stimulated conditions. (A-B) Data presented as percentage adhesion. (C-D) Data presented as fold change in adhesion which corrects for biological variability between batches. Fold adhesion data were chosen to be presented instead of adhesion percentage because of the variability between batches of mice and experiments. N=2-6 mice for both experiments. Error bars represent S.D.

To assess effector cytotoxic T lymphocyte adhesion to immobilized ICAM-1 we used a static adhesion assay, as previously. Milk was used to block unspecific protein binding in wells used as control, which yielded very low effector cytotoxic T lymphocyte adhesion (1.28%) as compared to immobilized ICAM-1 wells proving that effector cell adhesion was indeed dependent on integrin-ligand interactions. Stimulation of effector cytotoxic T lymphocytes from wild-type mice with phorbol ester or using anti-CD3 antibodies did not lead to a significant increase in cell adhesion to ICAM-1, suggesting that effector cytotoxic T lymphocytes do not need stimulation for activating β2-integrins for cell adhesion (Figure 37C).

Effector cytotoxic T lymphocytes also express β1, β3 and β7 integrins, such as VLA-4 integrins which binds to fibronectin to mediate cell adhesion (Shimidzu et al., 1990, Thatte et al., 2002, Humphries et al., 2006). Indeed, as shown in Figure 37D, effector cytotoxic T lymphocytes from wild-type mice can also adhere to fibronectin. Cell adhesion increased greatly (approximately 2.5 fold) when cells were stimulated with them chemical stimulant phorbol ester. ($p = 0.023$.) Phobol ester stimulation could thus activate integrin-mediated cell adhesion to fibronectin but not β2-integrin-mediated adhesion to ICAM-1 (Figure 37C and 37D). Although stimulation of effector cytotoxic T lymphocytes by anti-CD3 did lead to an increase in adhesion to fibronectin, this increase was not statistically significant (Figure 37B and 37D). Collectively, these data showed that β2-integrins were constitutively active in effector cytotoxic T lymphocytes while other (fibronectin-binding) integrins could be activated with phorbol ester in these cells.

4.3.3. Regulation of integrins in effector cytotoxic T lymphocytes.

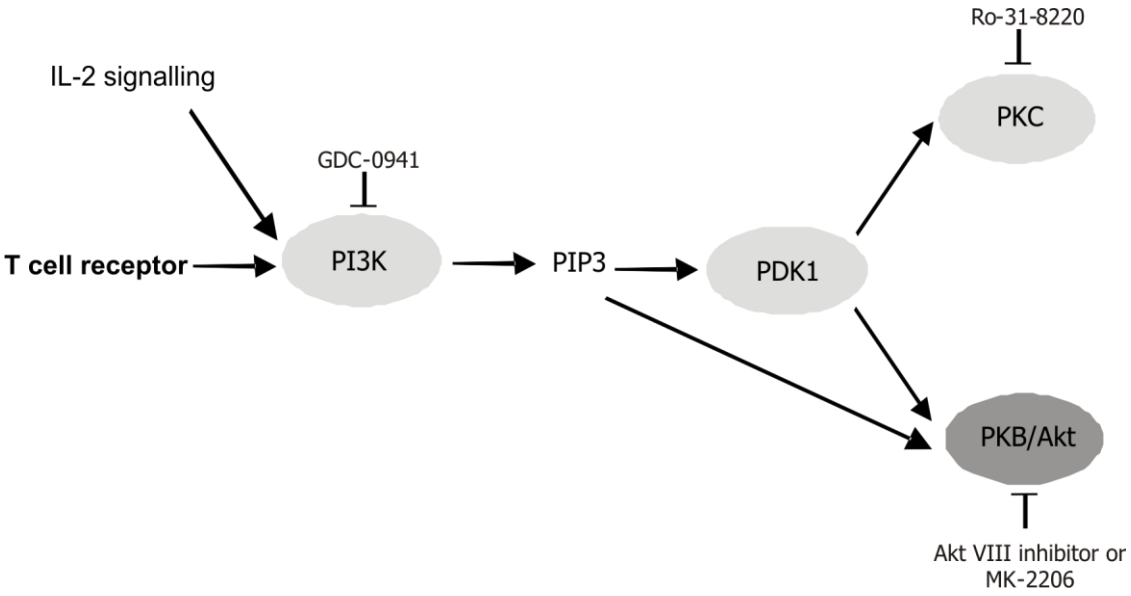


Figure 38. Schematic diagram depicting signalling pathways activated in effector cytotoxic T lymphocytes via T cell receptor and IL-2 signalling.

Effector cytotoxic T lymphocytes were established from spleen cells by anti-CD3 stimulation at the T cell receptor and maintained with IL-2 in medium for 7-10 days. IL-2 is secreted in large amounts by $CD4^+$ and $CD8^+$ T cells during an immune response and binds to the IL-2 receptor complex on the cell surface of T cells. The IL-2 receptor complex is made up of 3 subunits: the α chain (CD25), β chain (CD122 shared with IL-15 receptor) and γ chain (CD132 shared with IL-4, IL-7, IL-9 and IL-15 receptors) which are responsible for delivering growth proliferative signals (Boyman and Sprent, 2012). The lack of IL-2 from proliferating T cells can lead to cytokine withdrawal-mediated cell death (Cheng et al., 2002). IL-2 binding to the receptor will activate Janus kinase signal transducer JAK1 and JAK3, which in turn activates the STAT pathway which regulates gene transcription, Syk/Lck signalling (Zhou et al., 2000), mitogen-activated protein kinase (MAPK) pathway and other kinases such as MEK, MAPK/ERK, mTOR etc. IL-2 can also activate the Class 1A PI3K at the p85 subunit

(Zeiser and Negrin, 2008, Boyman and Sprent, 2012). Together with the activated T cell receptor, this PI3K activation leads to the production of PIP3 which directly activates Akt and also activates PDK1 that regulates other AGC kinases (such as PKC and Akt). Activation of these different signalling pathways in effector cytotoxic T lymphocytes may therefore have a role in inside-out signalling in the activation of integrins To study the role of these signalling pathways in regulation of integrin-mediated adhesion in effector cytotoxic T lymphocytes, inhibitors to inhibit the specific kinases such as PI3K, PKC and PKD were included in the study (Figure 38).

4.3.4. 2D migration of effector cytotoxic T lymphocytes

Akt inhibition in effector cytotoxic T lymphocytes results in reduced 2D migration on ligand.

Integrin-ligand interactions are needed for cell adhesion and migration. Effector cytotoxic T lymphocytes can use such interactions to migrate on surfaces. Compared to the smaller B cells, effector cytotoxic T lymphocytes often adopted a stretched out morphology and spread out onto surfaces with the formation of lamellipodia at the leading edge (Hogg et al., 2003, Smith et al., 2003, Evans et al., 2011) (Figure 39). To determine if motility and migration of effector cells was affected by concentration of integrin ligands, various concentrations of the LFA-1 ligand, ICAM-1 was immobilized on IBIDI plastic slides and effector cells from wild-type mice were left to interact with them for 30 minutes. The movement of cells were then recorded for up to an hour using time-lapse microscopy.

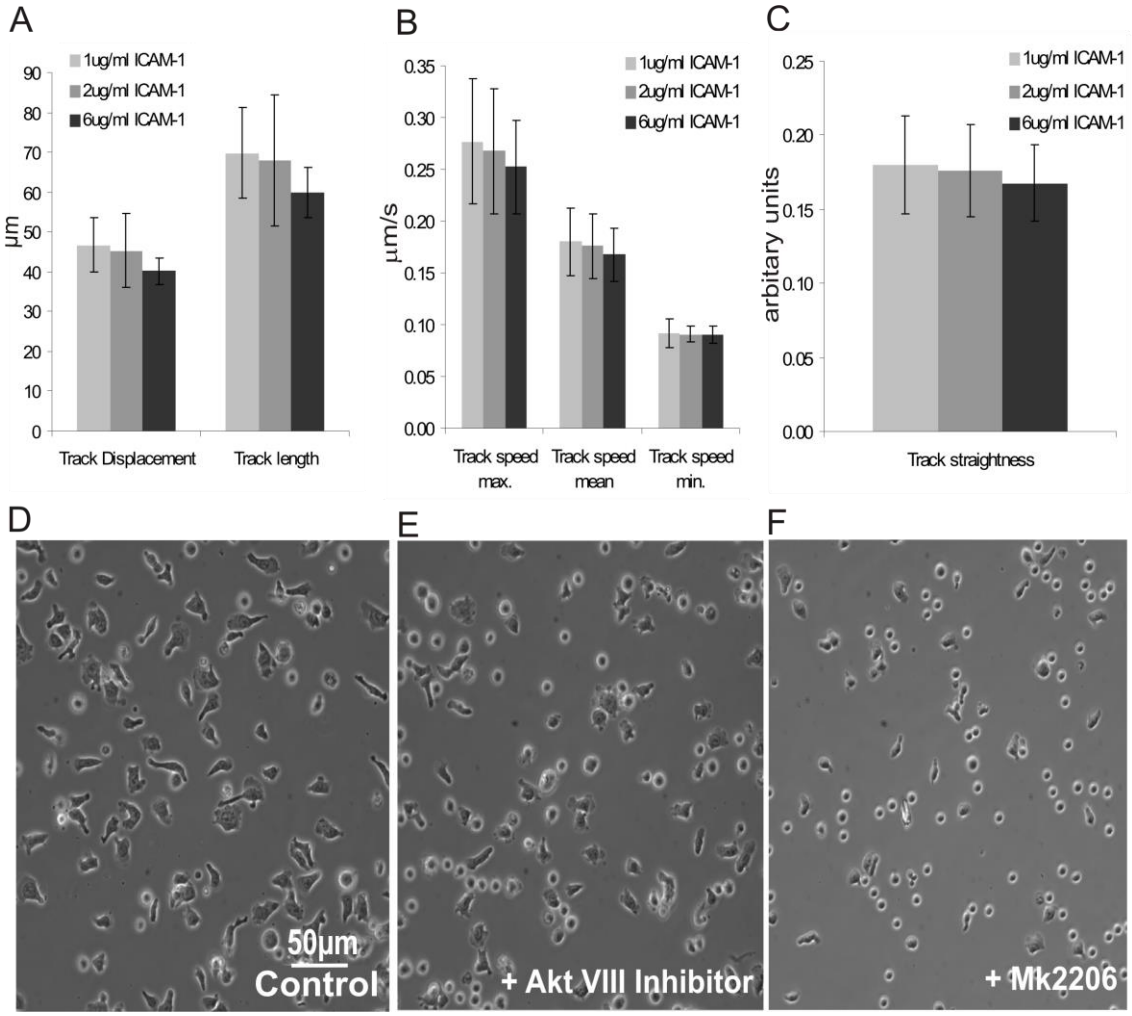


Figure 39. Effector cytotoxic T lymphocytes have the ability to migrate randomly and spontaneously on ICAM-1 and Akt inhibition results in reduced 2D migration abilities.

(A) Analysis of total displacement and length of distance travelled by effector cytotoxic T lymphocytes over surfaces with different concentrations of immobilized ICAM-1. (B) Analysis of maximum, mean and minimum track speed of effector cytotoxic T lymphocytes over surfaces with different concentrations of immobilized ICAM-1. (C) Analysis of track straightness travelled by effector cytotoxic T lymphocytes over surfaces with different concentrations of immobilized ICAM-1. For Figures A-C, error bars represent standard deviation, n = 8-10 experiments. P>0.05, Student's t-test. (D) Still image of untreated effector cytotoxic T lymphocytes interacting with 6 µg/ml of immobilized ICAM-1. (E) Still image of Akt VIII inhibitor-treated effector cytotoxic T lymphocytes interacting with 6µg/ml of immobilized

ICAM-1. (F) Still image of MK2206 inhibitor-treated effector cytotoxic T lymphocytes on surfaces with 6 µg/mlof immobilized ICAM-1. For Figures D-F, cells were viewed by microscopy at 40x, using phase-contrast 60 minutes after the start of the experiment.

Effector cytotoxic T lymphocytes were able to travel across surfaces randomly at about 60-70 µm/hour (Figure 39A). Maximum, mean and minimum speeds of effector cytotoxic T lymphocyte migration were also not affected by varying the concentrations of ICAM-1 on the surfaces (Figure 39B). Track straightness was determined by ratio of displacement travelled over distance where the value 1 represents absolute straightness. The data obtained show that effector cytotoxic T lymphocytes migrate randomly at approximately 0.16 in terms of straightness and this was not significantly affected by lowering ICAM-1 concentrations (Figure 39C). The effector cytotoxic T lymphocytes adopted a stretched out morphology with the formation of lamellipodia during the migration across the ICAM-1 layer (Figure 39D).

In the previous chapter, it was shown that the PI3K/Akt pathway was necessary for integrin-mediated cell adhesion in B cells. Interestingly, it was found that PI3K and Akt also significantly affected effector cytotoxic T lymphocyte adhesion to ICAM-1. Effector cytotoxic T lymphocytes treated with PI3k inhibitors (GDC-0941 and LY294002) or Akt Inhibitors (Akt VIII inhibitor or MK2206) displayed significantly reduced adhesion to ICAM-1 when compared to untreated effector cytotoxic T lymphocytes. (Data not shown. Experiments carried out by Vicky Morrison.) To investigate the role of Akt in integrin regulation in effector cytotoxic T lymphocytes further, the motility and morphology of effector cytotoxic T lymphocytes on ICAM-1 in the presence of Akt inhibitors was investigated using microscopy. It was observed that the addition of Akt VIII inhibitor (Figure 39E) or MK2206 (Figure 39F) to the cells

caused a significant proportion of cells to lose contact with the substrate and round up, thereby preventing cell migration on the substrate. Atomic Force Microscopy measurements comparing force and work needed to detach effector cytotoxic T lymphocytes pre-treated with Akt VIII inhibitors also showed that these cells have weaker adhesion to ICAM-1 ligands compared to untreated effector cytotoxic T lymphocytes. (Data not shown, experiment performed by Michael Conneely and Vicky Morrison.) Together, these results provided evidence that effector cytotoxic T lymphocyte integrin-mediated adhesion and migration on ICAM-1 in static conditions is dependent on Akt.

4.3.5. Adhesion of effector cytotoxic T lymphocytes in shear flow conditions

Development of physiologically relevant methods to study effector cytotoxic T lymphocyte adhesion under shear flow conditions.

Shear flow experiments have been used for investigation of adhesion of primary leukocytes and cell lines. This relatively new method has not been extensively developed and the set-up conditions that are necessary for this type of assay to assess primary murine effector cytotoxic T lymphocyte adhesion under flow have not been previously established. Therefore, we set out to develop shear flow adhesion assays for effector cytotoxic T lymphocytes.

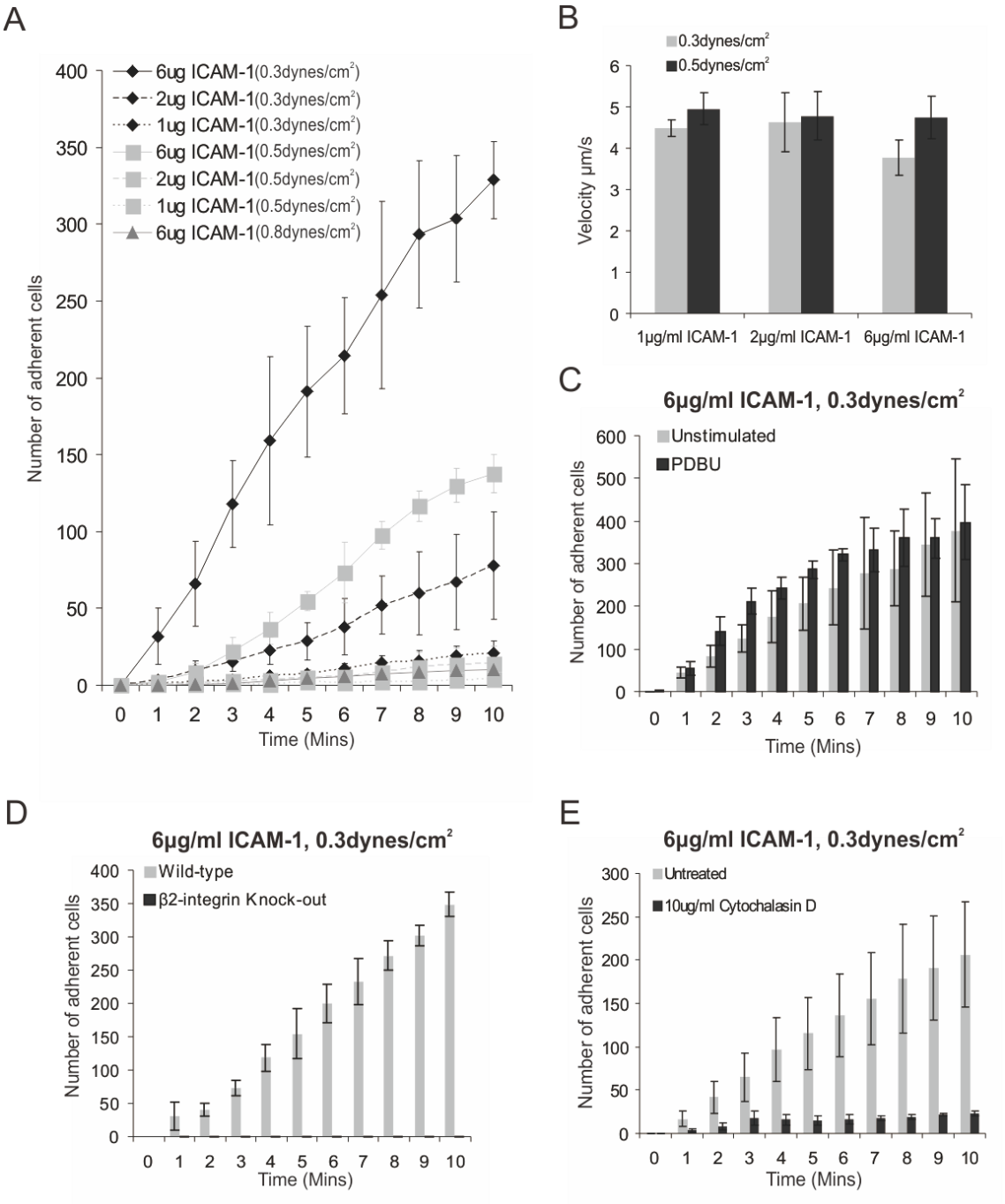


Figure 40. Setting up shear flow experimental conditions for effector cytotoxic T lymphocytes.

(A) Adhesion of effector cytotoxic T lymphocytes to ICAM-1 ligand under different shear stress rates. N=3. (B) Comparison of cell rolling rates across different ICAM-1 concentration under various shear stress rates. N=5. p>0.05 (ANOVA) (C) Assessing adhesion of effector cytotoxic T lymphocyte under shear stress of 0.3 dynes/cm^2 with and without cell stimulation by phorbol ester. N=3. p>0.05 (ANOVA). (D) Adhesion of effector cytotoxic T lymphocytes derived from

wild-type mice spleens or β2-integrin knock-out mice spleens under shear stress of 0.3 dynes/cm^2. N=2. p<0.05 (ANOVA). (E) Shear flow adhesion assay was carried out with effector cytotoxic T lymphocytes untreated or pre-treated with cytochalasin D at 10 µg/ml for 30 minutes at 0.3 dynes/cm^2. N =4. p<0.05 (ANOVA). Error bars represent S.D for all.

The concentration of ICAM-1 used for adhesion of B cells in static and shear conditions was 6 µg/ml. This was also the concentration of ICAM-1 ligand used in static adhesion assays with effector cytotoxic T lymphocytes. Adhesion assays with lower concentration of ligands showed that adhesion of effector cytotoxic T lymphocytes was indeed specific for ICAM-1 and LFA-1 interactions. The shear stress that was used for the B cell experiments was typically 0.3 dynes/cm^2 and could be increased up to 0.5 dynes/cm^2 for splenocytes. Effector cytotoxic T lymphocytes were able to adhere to immobilized ICAM-1 even at 0.5 dynes/cm^2 without stimulation. Increasing the shear stress in these experiments resulted in the reduction in the number of cells adhered to ICAM-1 (Figure 28 and 40A). Cells that were rolling across the surface slowly were selected and the velocity of these cells was quantified and there was no significant difference in rolling rates due to ICAM-1 interactions when ICAM-1 concentrations were varied, or when shear stressed was increased from 0.3 dynes/cm^2 and to 0.5 dynes/cm^2 (Figure 40B).

As shown in Figure 40A, adhesion of effector cytotoxic T lymphocytes did not require any cell stimulation under shear flow as high number of effector cells were able to adhere to ICAM-1 under these conditions (Figure 40A). Stimulating effector cytotoxic T lymphocytes with phorbol ester did not significantly increase adhesion of cells to ICAM-1 under shear flow (Figure 40C). The adhesion of effector cytotoxic T lymphocytes was specific as adhesion of effector cytotoxic T lymphocytes generated from β2-integrin knock-out mice spleens was severely compromised (Figure 40D).

These results show that effector cytotoxic T lymphocytes do not require stimulation to activate β2-integrin via inside-out signalling and an intact actin cytoskeleton for adhesion to ICAM-1 under flow.

As observed in 2D migration of effector cytotoxic T lymphocytes on ICAM-1 in static conditions, effector cytotoxic T lymphocytes actively crawl on ICAM-1 and change their morphology as they travel across surfaces (Figure 39D-39F). This suggested that the actin cytoskeleton played an important part in effector cytotoxic T lymphocytes migration and could contribute to resisting shear flow forces in integrin-mediated adhesion. Indeed, effector cells pre-treated with cytochalasin D (a disruptor of actin polymerization) were significantly less able to bind to ICAM-1 under shear flow (Figure 40E).

Adhesion of effector cytotoxic T lymphocytes to endothelial cells under shear flow.

Endothelial cells lining the blood vessels are exposed to shear stress caused by blood flow and the shear stress present causes endothelial cells to elongate, align to the direction of flow, form thicker stress fibres and display increased expression of ICAM-1 (Tzima et al., 2003). In order to be able to observe integrin-mediated adhesion of lymphocytes at a more physiologically relevant level, mouse ICAM-1 expressing endothelial cell line from cortex/brain, bEnd.3 was grown in Ibidi slides. These cells were grown for 6-8 days at 15 dynes/cm^2 if polarised cell layers were needed, or alternatively, grown in static conditions for 2 days.

For assays using B cells or $CD4^+$, $CD8^+$ T cells, SDF-1 was then applied onto the endothelial cell layers for the immobilized SDF-1 to stimulate cell adhesion. (Data not shown.) However, as shown in Figure 40, effector cytotoxic T lymphocytes did not require inside-out signalling to trigger adhesion to an ICAM-1 layer. Therefore, the effector cytotoxic T lymphocytes were able to adhere to bEnd.3 layers without cell stimulation.

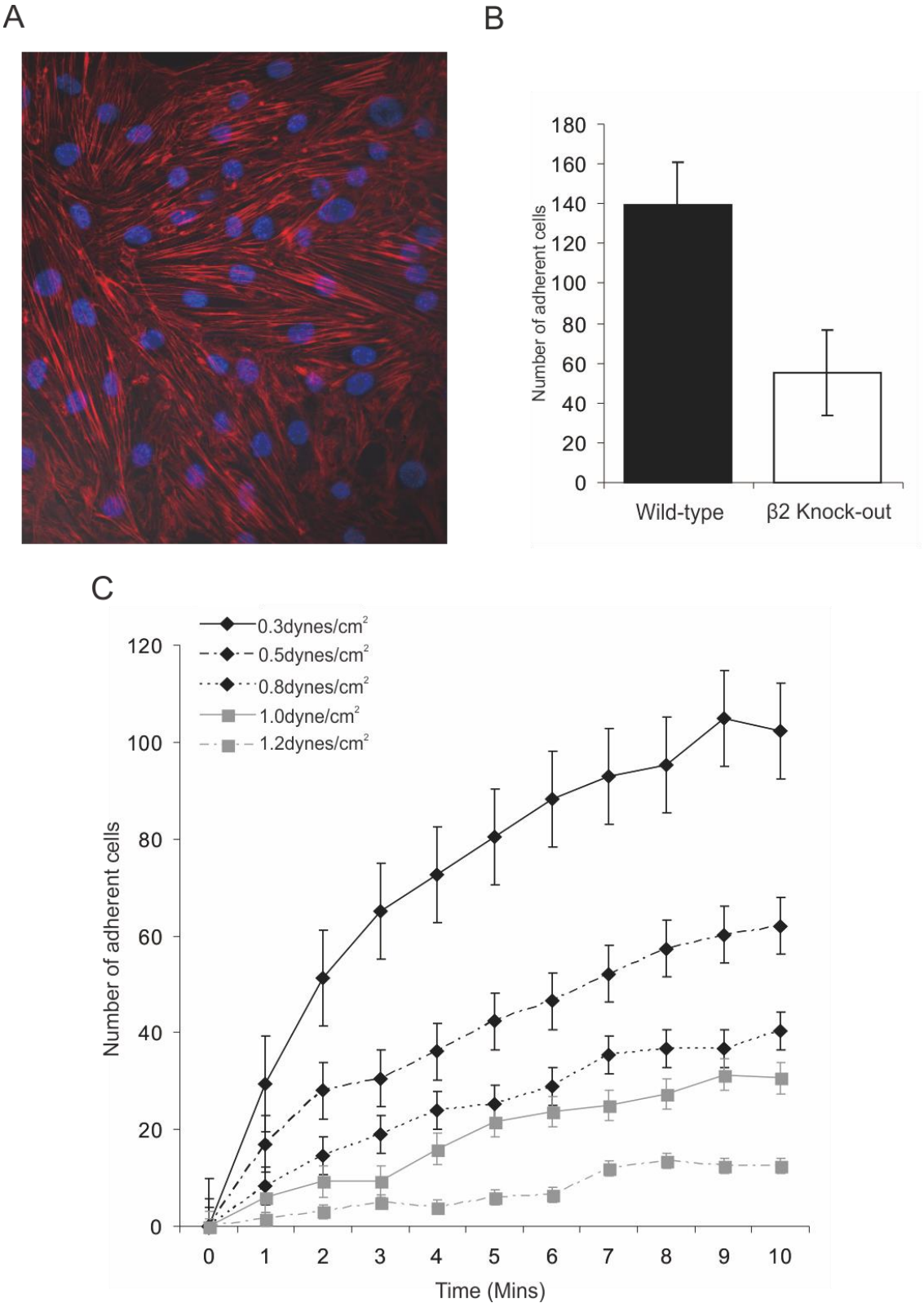


Figure 41. Shear flow experiments of effector cytotoxic T lymphocytes using bEnd.3 layers.

(A) bEnd.3 cell layers were stained with phalloidin-red for the detection of actin and stained with DAPI-blue for detection of cell nuclei. A representative image is shown, N=5. (B)

Comparing the adhesion levels of wild-type and β2-integrin knock-out effector cytotoxic T lymphocytes binding to bEnd.3 endothelial cells under shear flow conditions at 0.3 dynes/cm^2. The number of adhered effector cytotoxic T lymphocytes at the 8 minutes end time-point was counted. N=3 pairs. Error bars are S.D. (C) Adhesion of effector cytotoxic T lymphocytes onto bEnd.3 endothelial cells at various shear stress rates. N=3, error bars are S.E.

bEnd.3 cells constitutively express ICAM-1, MAd-CAM-1 and VCAM-1. After growing bEnd.3 layers, TNF-α was added to the growth medium to stimulate E-selectin and P selectin expression and increase ICAM-1 expression (Sikorski et. al., 1993). These cells were able to grow on the IBIDI-channels used in shear flow assays and adopted an elongated structure with actin stress fibres (Figure 41A). Effector cytotoxic T lymphocyte adhesion to bEnd.3 endothelial cell layer was heavily dependent on LFA-1 integrin binding to ICAM-1, as β2-integrin knock-out effector cytotoxic T lymphocytes displayed significantly lower adhesion to bEnd.3 cells compared to wild-type cells at 0.3 dynes/cm^2 (Figure 41B). The shear stress could be increased to 1 dyne/cm^2 in these assays, which resulted in approximately 30 effector cells adhering to the endothelial cell layer (Figure 41C). Effector cytotoxic T lymphocytes could still adhere to bEnd.3 cells at higher shear stress of 1.2 dynes/cm^2. In addition, there were more cells adhering to endothelial cell layers than to immobilized ICAM-1 at the lower shear stress of 0.8 dynes/cm^2 (Figure 40A and 41C). This difference in adhesion may be due to the presence of additional adhesion molecules such as E-selectin on endothelial cells, which interact with and slow down leukocytes on the endothelial surfaces (Figure 40A and 41C). E-selectin has also been found to activate β2-integrin binding to ICAM-1 via signalling through mitogen-activated protein kinases (Simon et al., 2000, Green et al., 2004).

To test whether E-selectin contributes to effector cytotoxic T lymphocyte adhesion under shear flow conditions, E-selectin was co-immobilized with ICAM-1 on IBIDI channels and shear flow assays using varying shear stress rates were carried out (Figure 42).

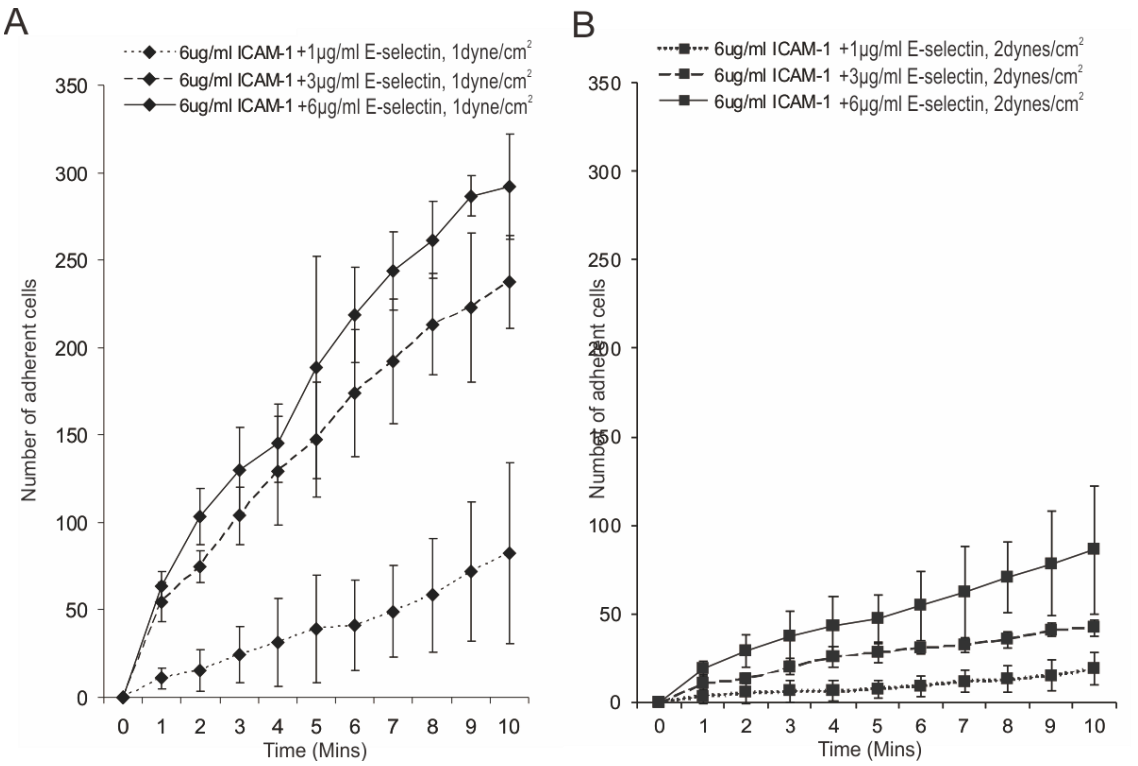


Figure 42. Effector cytotoxic T lymphocyte shear flow experiments using combinations of ICAM-1 and E-selectin.

The influence of various combination of E-selectin and ICAM-1 on effector cytotoxic T lymphocyte adhesion at (A) 1 dyne/cm^2 or at (B) 2 dynes/cm^2. N=3. Error bars represent S.D.

Indeed, the shear flow could be increased up to 2 dynes/cm^2 in these assays, which still resulted in significant cell adhesion, and subsequent experiments were carried out at 6 µg/ml ICAM-1 with 3 µg/ml E-selectin for high shear flow experiments (Figure 42). Shear flow assays without immobilized E-selectins were not carried out here as the

experiments conducted in Figure 41C has shown that at 1 dyne/cm^2, there was about 30 cells adhering to 6 μg/ml ICAM-1 without E-selectin. This is less than the 50 cells observed in the presence of 1 μg/ml E-selectin which supports the hypothesis that the presence of E-selectin increases cell adhesion under shear flow (Figure 41C and 42).

4.3.6. Role of AGC kinases in effector cytotoxic T lymphocyte adhesion under shear flow conditions

4.3.6.1. PI3K/Akt

Effector cytotoxic T lymphocyte adhesion to ICAM-1 under shear stress was affected by PI 3 kinase inhibition but not by Akt inhibition

In the previous chapter, PI3K and Akt were found to be necessary for the regulation of B cell adhesion under shear flow conditions. However, the role of the PI 3-kinase/Akt pathway in effector cytotoxic T lymphocyte integrin regulation under shear flow conditions has not been investigated.

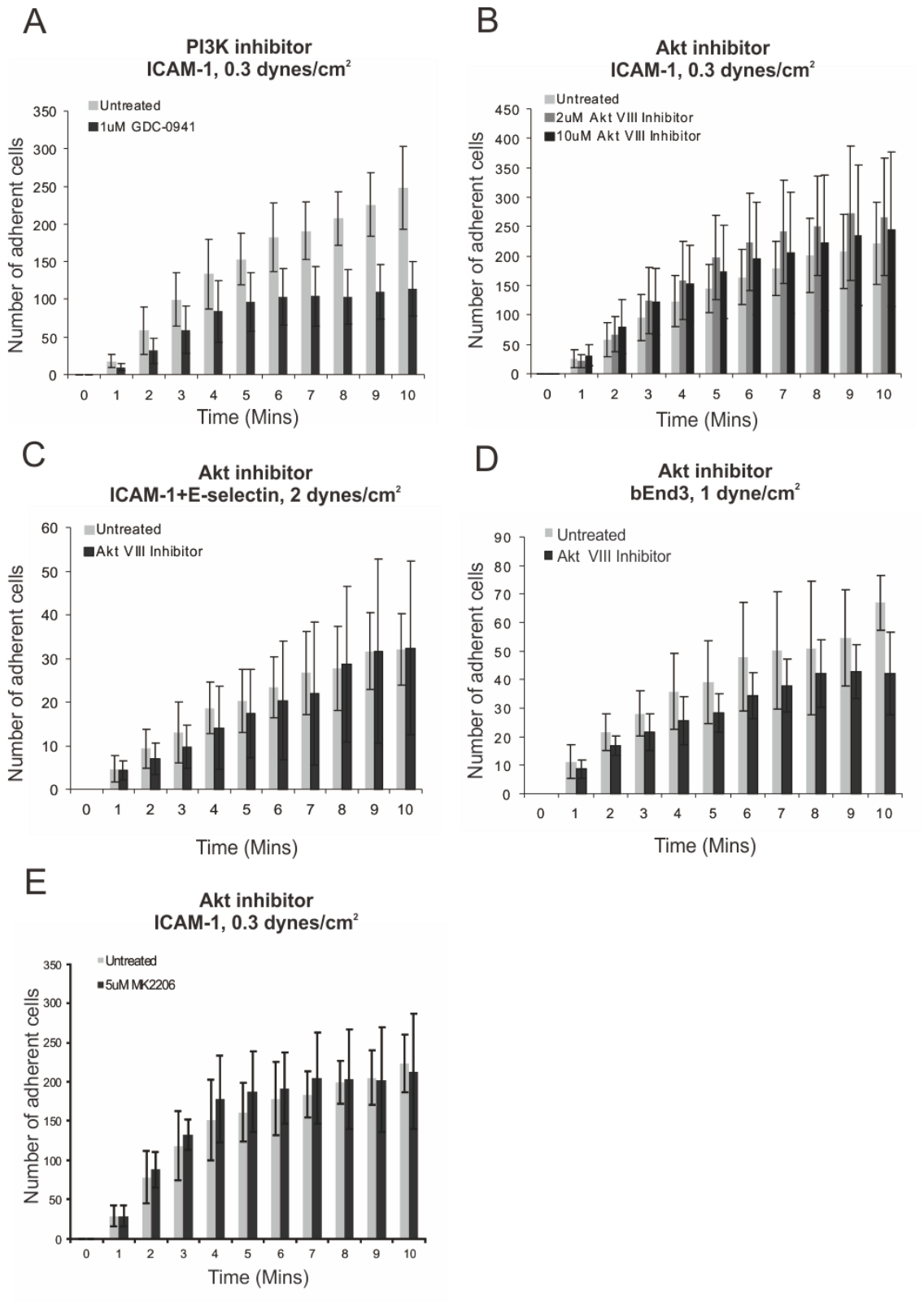


Figure 43. Effector cytotoxic T lymphocyte adhesion to ICAM-1 under shear flow was not dependent on Akt but was affected by PI 3-kinase inhibition.

(A) Adhesion assay under shear flow conditions at 0.3 dynes/cm^2 using untreated and PI 3-kinase inhibitor-treated (GDC-0941, 1 μM) effector cytotoxic T lymphocytes to ICAM-1. N=4, p<0.05 (ANOVA). (B) Adhesion of untreated, 2 uM and 10 μM Akt VIII inhibitor-treated effector cytotoxic T lymphocytes to ICAM-1 under low shear flow of 0.3 dynes/cm^2. N=3 and N=5 respectively, p>0.05 (ANOVA). (C) Adhesion of untreated and 10μM Akt VIII inhibitor-treated effector cytotoxic T lymphocytes to 6 μg/ml ICAM-1 with 3 μg/ml E-selectins under high shear flow of 2 dynes/cm^2. N=3, p>0.05 (ANOVA). (D) Adhesion of untreated and 10 μM Akt VIII inhibitor-treated effector cytotoxic T lymphocytes to bEnd.3 cells to stimulate endothelial blood vessels under high shear flow of 1 dyne/cm^2. N=3, p>0.05 (ANOVA). (E) Adhesion of untreated and MK2206 (another Akt inhibitor) inhibitor-treated (5 μM) effector cytotoxic T lymphocytes to ICAM-1 under shear flow at 0.3 dynes/cm^2.N=3, p>0.05 (ANOVA). In all cases error bars represent S.D.

With the introduction of shear stress in adhesion assays to mimic cell adhesion in blood vessels, effector cytotoxic T lymphocytes pre-treated with PI 3-kinase inhibitors showed significantly less adhesion compared to untreated cells (Figure 43A). GDC-0941 was used instead of LY294002 due to better specificity as discussed in Chapter 3, with reference to the publication from Bain et al., 2007. Adhesion assay results and western blot results in Figure 24 also suggested that GDC-0941 could be a more suitable PI 3-kinase inhibitor compared to LY294002. In contrast, the Akt VIII inhibitor did not affect effector cytotoxic T lymphocyte adhesion to ICAM-1 under shear flow at 0.3 dynes/cm^2 (Figure 43B). At higher shear stress of 2 dynes/cm^2 using ICAM-1 and E-selectin ligands, or to bEnd.3 layers at 1 dyne/cm^2, inhibition of Akt using Akt VIII inhibitor also did not significantly affect effector cytotoxic T lymphocyte adhesion under shear flow (Figure 43C and 43D). Similar results were observed using another Akt inhibitor, MK2206, to pre-treat effector cytotoxic T lymphocytes before adhesion to ICAM-1 ligands at low shear stress (Figure 43E). Western blot detection of

phosphorylated Akt (Thr308) levels was performed and it was confirmed that these PI 3-kinase and Akt inhibitors were effective in inhibiting Akt activity in these cells. (Data not shown, experiment performed by Vicky Morrison.) Taken together, these results show that PI3K but not Akt affects integrin regulation in effector cytotoxic T lymphocyte adhesion under shear flow conditions.

4.3.6.2. ADAP/Syk

Other known regulators of LFA-1 such as ADAP and Syk signalling did not affect effector cytotoxic T lymphocyte adhesion under shear flow.

ADAP and SLP-76 have been found to regulate LFA-1 in T cells in static conditions (Wang et al., 2009). Zap-70, which is upstream of ADAP and SLP-76, was also found to be one of the regulators of LFA-1 by activating Rap1 in lymphoblasts (Evans et al., 2011). However, we found that effector cytotoxic T lymphocytes where Syk/Zap-70 was inhibited or which lacked ADAP did not show reduced adhesion to ICAM-1 under shear flow, showing that ADAP and Syk/Zap70 are not important for integrin regulation under shear flow conditions in effector cytotoxic T lymphocytes (Figure 44A and 44B).

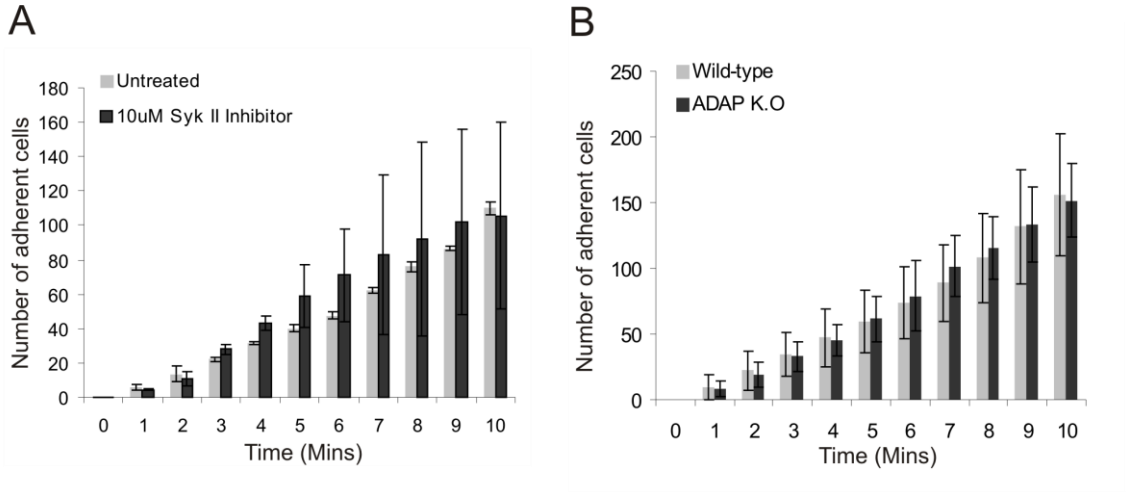


Figure 44. Integrin-mediated cell adhesion to ICAM-1 under shear stress was not dependent on ADAP/Syk signalling.

(A) Effector cytotoxic T lymphocytes that were untreated or pre-treated with 10 μM Syk II inh*ibitor for 1 hour were used in* shear flow adhesion assays to ICAM-1 at 0.3 dynes/cm2. *N=3. p>0.05 (ANOVA). (B) Effector cytotoxic T lymphocytes from wild-type mice spleens or ADAP-/- mice spleens used in shear flow adhesion assays to ICAM-1 at 0.3 dynes/cm^2. N=3. p>0.05 (ANOVA).*

4.3.6.3. PLC and Calcium signalling

Effector cytotoxic T lymphocytes did not require PKC for integrin-mediated cell adhesion under shear flow.

It has recently been shown that human effector cytotoxic T lymphocytes do not require chemokine-induced inside-out signalling for adhesion under shear flow and that PLCγ activity is necessary for effector cytotoxic T lymphocyte adhesion via outside-in signalling under these conditions (Shulman et al., 2012). Although PLC signalling has been shown to be essential for inside-out signalling in human primary T cells,

downstream effectors of PLC signalling important for effector cytotoxic T lymphocyte adhesion under shear flow have not been identified (Patsoukis et al., 2009). PLCγ activates PKC via increased production of diacylglycerol (Figure 45). In addition, PLC activation leads to release of calcium, which can activate other calcium dependent signalling pathways (Letschka et al., 2008). Therefore, the potential involvement of these signalling pathways in effector cytotoxic T lymphocyte adhesion under shear flow was next investigated.

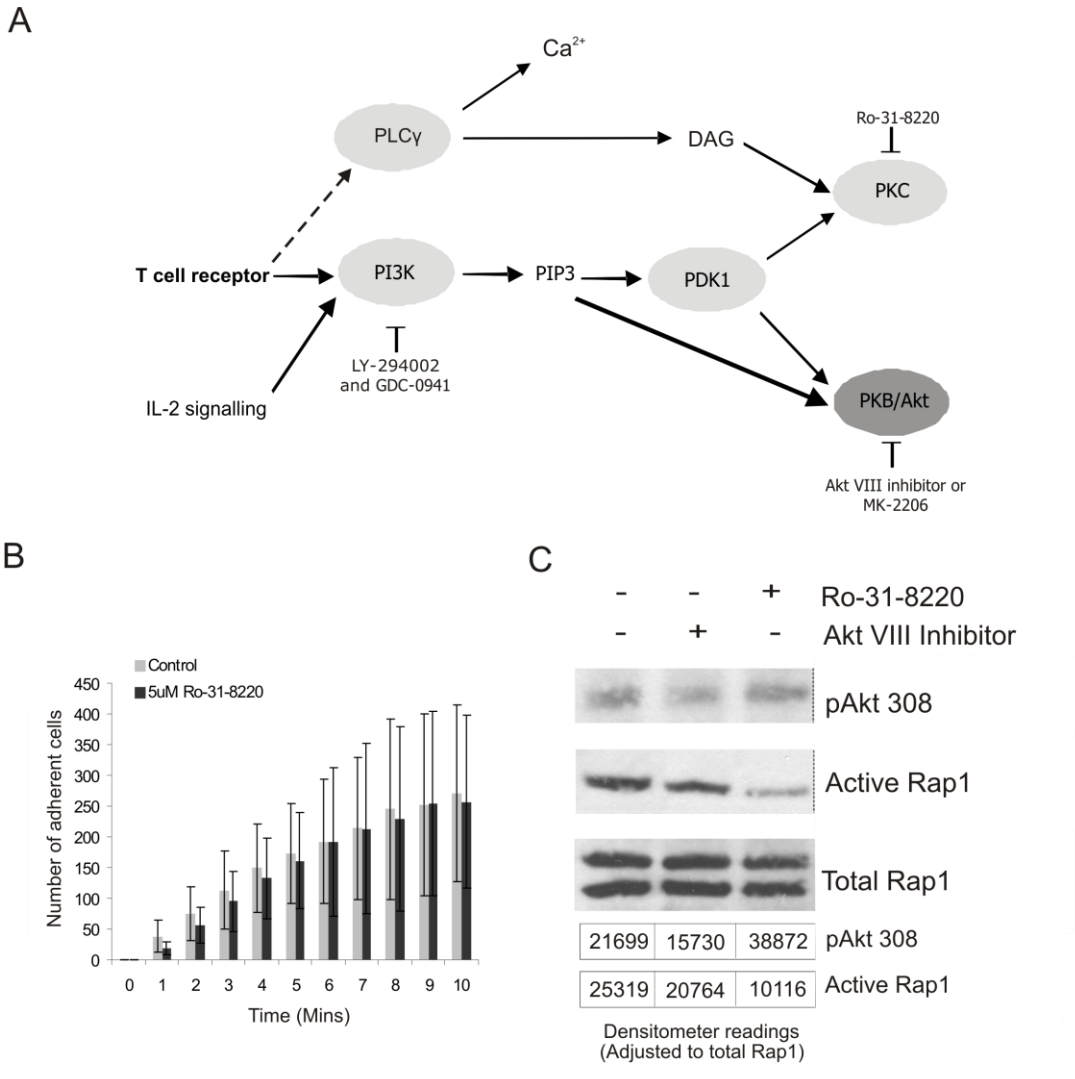


Figure 45. Inhibition of PKC in effector cytotoxic T lymphocytes did not affect adhesion to ICAM-1 under shear flow.

((A) Schematic representation of T cell signalling after T cell receptor activation or by IL-2 in effector cytotoxic T lymphocytes. Aside from activating the PI3K/Akt pathway, triggering of the T cell receptor activates Phospholipase C signalling, which leads to the release of calcium ions and increase of diacyglycerol which activates PKC. (B) Effector cytotoxic T lymphocytes were untreated or pre-treated with PKC inhibitor 5 µM Ro-31-8220 and adhesion to ICAM-1 was assessed at 0.3 dynes/cm^2. N=3. p>0.05 (ANOVA). (C) Western Blot detection of phosphorylated Akt (Thr308), active Rap1 and total Rap1 levels present in cell lysates. Effector cytotoxic T lymphocytes were untreated or pre-treated with PKC inhibitor Ro-31-8220 or Akt inhibitor Akt VIII inhibitor. Levels of phosphrylated Akt (Thr308), Active Rap1 and total Rap1 were detected in effector cytotoxic T lymphocyte lysate and densitometer readings of pAkt 308 levels and active Rap1 levels were normalised to the same level of total Rap1. Results representatiove of 1 experiment. N=3.

PKC regulates activation of integrins in B cells under both static and shear flow conditions (Chapter 3). However, PKC inhibition did not affect effector cytotoxic T lymphocyte adhesion to ICAM-1 under shear flow conditions (Figure 45B). This result excluded the involvement of PKC in regulation of effector cytotoxic T lymphocyte adhesion under shear flow conditions.

Integrin-mediated adhesion under flow is independent of Akt and Rap1

In Chapter 3 it was found that Akt may control Rap1 upstream of integrin activation in B cells. Whether PI3K or Akt regulate Rap1 in effector cytotoxic T lymphocytes was not known and was investigated by detection of active Rap1 levels by pulldown assays, as previously. Resting effector cytotoxic T lymphocytes had a high level of active Rap1. The Akt VIII inhibitor used at 10 µM had been shown to decrease phosphorylated Akt

(Thr308) levels in effector cytotoxic T lymphocytes as shown by western blot analysis but did not affect active Rap1 levels (Figure 45C). With the results from shear flow adhesion assays in Figure 43, these suggested that unlike in B cells, Akt inhibition does not affect Rap1 and Rap1 does not affect adhesion of effector cytotoxic T lymphocytes. On the other hand, inhibition of PKC using Ro-31-8220 significantly decreased active Rap1 levels in preliminary experiments. The effect of PKC inhibitors on Rap1 activity may be due to an active PKCθ/RapGEF2 pathway that has previously been reported in T cells (Letshcka et al., 2008). As Ro-31-8220 did not inhibit adhesion under flow, but did inhibit Rap1, these results also indicate that Rap1 does not regulate adhesion of effector cytotoxic T lymphocytes under shear flow conditions (Figure 45).

Calcium-binding calmodulin was necessary in the regulation of effector cytotoxic T lymphocyte adhesion under shear flow.

In addition to PKC activation, PLC signalling generates intracellular calcium signals which could potentially activate many other intracellular processes during T cell activation (Kim et al., 2009) (Figure 45A). It was therefore important to investigate if intracellular calcium signals affected adhesion of effector cytotoxic T lymphocyte under shear flow conditions (Figure 46).

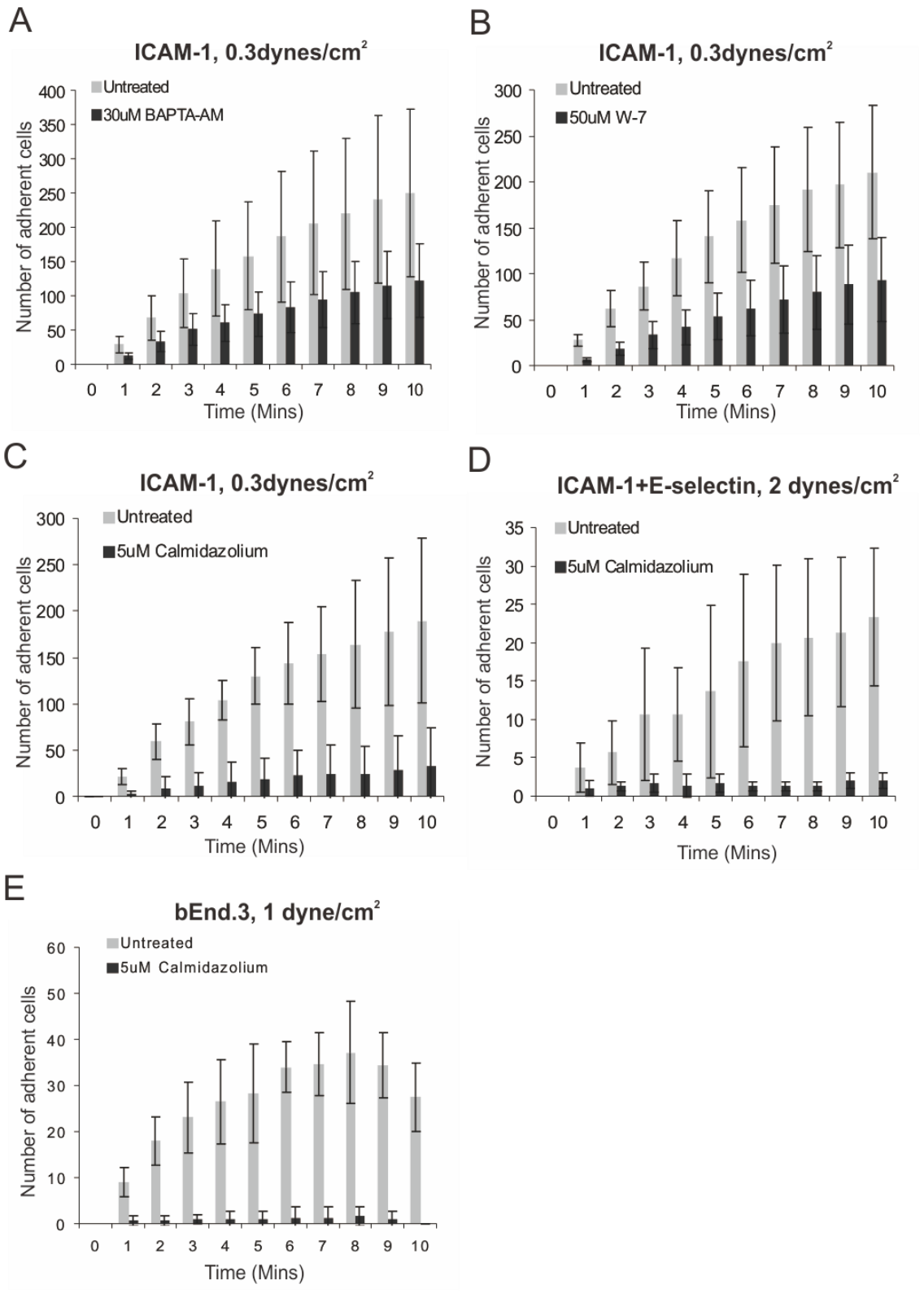


Figure 46. Integrin mediated effector cytotoxic T lymphocyte adhesion to ICAM-1 under flow is dependent on calcium/calmodulin.

(A) Effector cells treated with 30 µM BAPTA-AM in PBS to remove intracellular calcium were allowed to adhere to 6 µg/ml ICAM-1., N=4. p>0.05, (ANOVA). Error bars represent S.E.M

(standard error of mean). (B) Effector cells treated with W7 calmodulin-inhibitor in PBS adhering to 6 µg/ml ICAM-1. N=3. p<0.05 (ANOVA). Error bars represent S.E.M. (C) Effector cells treated with Calmidazolium calmodulin-inhibitor in PBS adhering to 6 µg/ml ICAM-1. N=3. p<0.05, (ANOVA). Error bars represent S.D. (D) Effector cells treated with Calmidazolium in PBS adhering to 6 µg/ml ICAM-1 and 3 µg/ml E-selectin at high shear stress of 2 dynes/cm^2. N=3. p<0.05 (ANOVA). Error bars represent S.D. (E) Effector cells treated with Calmidazolium in PBS adhering to bEnd3 to stimulate blood vessel conditions at 1 dyne/cm^2. N=3. p<0.05, (ANOVA). Error bars represent S.D.

Experiments investigating the role of calcium in effector cytotoxic T lymphocyte adhesion were carried out in PBS as RPMI contains small amounts of calcium which could interfere with the assays. A cell-permeant chelator which is more selective for calcium than magnesium, BAPTA-AM, was first used to treat effector cells and control intracellular calcium levels. Effector cytotoxic T lymphocytes treated with BAPTA-AM were less adhesive to ICAM-1 compared to untreated cells, indicating that calcium signalling was necessary for effector cytotoxic T lymphocyte adhesion under shear flow. (Figure 46A. Experiments carried out by Hwee San Lek and Vicky Morrison.)

Calmodulin is an important calcium binding protein that mediates various calcium-mediated cellular responses and can be inhibited by using inhibitors W-7 or calmidazolium. Effector cytotoxic T lymphocytes pre-treated with the membrane permeable W-7 calmodulin antagonist had significantly lower cell adhesion compared to control effector cytotoxic T lymphocytes (Figure 46B). (Experiments carried out by Hwee San Lek and Vicky Morrison.) Cells pre-treated with calmidazolium were also significantly affected in cell adhesion to ICAM-1 under low shear stress (Figure 46C). Similar results were observed at higher shear stress of 2 dynes/cm^2, when the cells were allowed to adhere to ICAM-1 and E-selectin (Figure 46D). This result was also

observed when shear flow experiments comparing untreated or calmidazolium-treated effector cytotoxic T lymphocytes adhering to endothelial cell layers at 1 dyne/cm^2 were carried out (Figure 46E). Calmidazolium is not only a calmodulin inhibitor but also a calcium channel inhibitor, which could explain why this inhibitor was more effective in inhibiting effector cytotoxic T lymphocyte adhesion. These results from experiments using BAPTA-AM, W-7 and Calmidazolium indicate that calcium signalling and calmodulin were important in regulation of LFA-1 in effector cytotoxic T lymphocyte adhesion to ICAM-1 under shear flow.

4.4. Discussion

Here, we have examined how effector cytotoxic T lymphocyte integrin-mediated adhesion is regulated. Effector cytotoxic T lymphocytes expressed much higher levels of β2-integrins compared to positively-selected CD4$^+$ and CD8$^+$ T cells (Experiments carried out by Vicky Morrison.). These higher levels of integrins may contribute to adhesion to ICAM-1 ligands in both static and shear flow conditions. It was observed that LFA-1 integrins on effector cytotoxic T lymphocytes did not require stimulation by phobol ester or via CD3 to mediate cell adhesion to ICAM-1 (Figure 37C). Therefore, effector cytotoxic T lymphocytes in tissues may have active LFA-1 on their surfaces, which bind to ICAM-1 on other cells to enhance signalling between two cells. For example, CD8$^+$ effector cytotoxic T lymphocytes may use constitutively active LFA-1 facilitate killing of infected cells. Effector cytotoxic T lymphocytes were also shown to be able to migrate spontaneously on ICAM-1 without chemokine stimulation (Figure 39), which suggested that effector cytotoxic T lymphocytes can freely migrate on endothelial cells and in tissues, which may further facilitate immune responses.

In static conditions, Akt was also found to inhibit adhesion of cytotoxic T cell adhesion to ICAM-1. (Data not shown. Experiments carried out by Vicky Morrison.) However, active Rap1 levels were not affected when Akt was inhibited (Figure 46). How does Akt regulate effector cytotoxic T lymphocyte migration on ICAM-1 in static conditions? Effector cytotoxic T cells showed significant increased in adhesion to fibronectin when stimulated with phorbol ester but no significant increase in adhesion to ICAM-1 in static conditions, suggesting that different integrins, such as VLA-4 (which can bind to fibronectin) could potentially be activated for cell adhesion (Figure 38). How effector cytotoxic T cells regulate VLA-4 adhesion to VCAM-1, if Akt has any influence in VLA-4 regulation and if these cells switch between LFA-1 and VLA-4 in the presence of shear stress could be investigated in future. In addition, L-Plastin is a regulator of F-actin at the leading edge of cells and is phosphorylated by PKCζ, which is an atypical PKC isoform, in human T lymphocytes during chemotaxis. L-plastin was also suggested to regulate SDF-1α mediated lymphocyte migration on ICAM-1, independent of LFA-1 activated adhesion. Knockdown of L-plastin in human lymphocytes was observed to impair the Rac1 activation cycle and Akt phosphorylation in response to SDF-1α stimulation (Freeley et al., 2012). Soriano et. al., (2011) also suggested that uropod contractility was necessary for transendothelial migration. Sharpin has also been recently suggested to be an inhibitor of β1-integrins which regulates uropod contractility (Pouwels et al., 2013). It is possible that Akt inhibition affects myosin and/or sharpin in uropod contractility, thereby influencing 2D migration of effector cytotoxic T lymphocytes on ICAM-1 (Smith et al., 2003, Pouwels et al., 2013) (Figure 39).

Freeley et al. also observed that L-plastin knock-out effector cytotoxic T lymphocytes have impaired Akt phosphorylation, but were able to adhere to and migrate on endothelial cells normally under shear flow conditions (Freeley et al., 2012). This

supports the results observed in Figure 43 and further enhanced the importance of our findings that calcium-binding calmodulin signalling is more relevant in controlling effector cytotoxic T lymphocyte adhesion under flow. Future investigations using PKCζ inhibitors or cells lacking PKCζ could be carried out to determine if PKCζ influences integrin–mediated 2D migration in effector cytotoxic T lymphocytes. These models could also be used in adhesion assays under shear flow conditions to investigate the role of this kinase in effector cytotoxic T lymphocytes adhesion under shear flow.

It is important to study of how adhesion of effector cytotoxic T lymphocytes in the presence of shear flow is regulated. Effector cytotoxic T lymphocytes have to resist shear flow to traffic into sites of inflammation in tissues. The high surface levels of integrins in effector cytotoxic T lymphocytes appeared to be important in cell adhesion and migration, as these cells displayed ability to resist shear flow without needing chemokine or phorbol ester stimulation, unlike, for example, B cells (Figure 37C and 40C). The results shown in Figures 40A-40C are similar to those presented by Shulman et al., (2012), where the authors reported that effector T lymphocytes (both $CD4^+$ and $CD8^+$) did not require chemokines for the activation of integrins to mediate adhesion under shear flow. However, the adhesion assays used by our groups differed from theirs as they were interested in effects of outside-in signalling, which emphasize on signalling events after integrin is bound to ligands and the experiments were done using effector T cells cultured from human peripheral blood. We are interested in effector cytotoxic T lymphocytes that are captured by integrins alone or through integrin/selectin bonds under shear flow conditions. Therefore in our assays, effector cytotoxic T lymphocytes were introduced into flow channels under shear flow conditions. In contrast, Shulman et. al. allowed cells to settle and bind to the ligands first allowing outside-in signalling to occur before shear flow was introduced (Shulman et al., 2012).

Therefore, as our assay uses shear flow conditions also during the capture phase, it more closely mimics what is happening in shear flow conditions in vivo, and we have shown here for the first time that effector cytotoxic T lymphocytes can adhere to coated ICAM-1 and E-selectin under shear flow conditions without chemokine-stimulation.

Valignat et al, 2013 showed that shear stress affected directionality of effector T cells and primary T cell migration instead of speed of cell migration. It was also shown that that use of T cell line HSB2 did not have the same effect under shear stress, which emphasized the importance of conducting such cell adhesion and migration studies using primary T cells. Neutrophils also acted differently from T cells by crawling in the direction of shear flow instead of against the shear flow as demonstrated by primary and effector T cells which supports the hypothesis that regulation of leukocyte cell adhesion and migration under shear flow could be different in different cell types (Valignat et al., 2013).

We show here that E-selectin was required for effector cytotoxic T lymphocyte adhesion under shear flow conditions at higher shear flow rates. ICAM-1 alone allowed cell capture at shear stress up to 0.5 dynes/cm^2 but 0.3 dynes/cm^2 was used for most assays, as there was less variability in the numbers of adhered effector cytotoxic T lymphocytes from different mice using lower shear rates. A combination of E-selectin and ICAM-1 allowed cell capture of effector cytotoxic T lymphocytes to occur under high shear stress of 2 dynes/cm^2. In this scenario, PSGL-1 and CD43 on effector cytotoxic T lymphocytes interact with E-selectins and this increase in interactions between adhesion molecules leads to slow rolling of cells on the surface of the channel. L-selectins (CD62L) are constitutively expressed on leukocytes. For example, at the start of the extravasation process, polymorphornuclear leukocytes (PMNs) use L-

selectin to tether along the endothelial walls while making brief contacts with the ligands such as GlyCAM-1, PSGL-1, CD34 and mADCAM-1 (Nicholson et al., 1998). This rolling process is also supported by binding to E-selectin (CD62E) and P-selectin (CD62P) found on endothelial walls, which interacts with PSGL-1 expressed on leukocytes (Rahman and Fazal, 2009). Rolling mediated by selectins allows more time and contact for LFA-1 integrins to mediate strong adhesion for cell arrest in the next stage of the extravasation progress (Tedder et al., 1995, Matsumoto et al., 2005). ICAM-1 expression is upregulated on the surfaces of endothelial ell walls during inflammation. Activated β2-integrins on the surface of leukocytes can then bind to ICAM-1 which causes cell arrest (Rahman and Fazal, 2009).

Although bEnd.3 endothelial cell layers express a more complex combination of adhesion molecules that can interact with effector cytotoxic T lymphocytes, this did not necessarily translate to an ability to capture cells at higher shear stress. Possibly the cells were not grown for long enough to obtain polarised, elongated cells and hence the expression of adhesion molecules was not fully optimised. Alternatively, other interactions of the endothelial cell layer negatively regulate the adhesion process. In addition, the combination of co-immobilized E-selectin and ICAM-1 ligands in vitro was optimised for observing LFA-1-ICAM-1 cell adhesion. These molecules may not have been expressed at such high concentrations on endothelial cells.

An important observation was that static and shear flow adhesion of effector cytotoxic T lymphocytes appeared to be differentially regulated by Akt, downstream of PI 3-kinase. When Akt was inhibited in effector cytotoxic T lymphocytes, adhesion was reduced under static but not in shear flow conditions. Preliminary data from mature T cells lacking PDK1 (upstream of Akt) showed that this kinase plays a role in integrin

regulation in T cells. Although integrin expression at the cell surface was not affected by deletion of PDK1, adhesion to fibronectin and ICAM-1 was affected in PDK1 knock-out effector cytotoxic T lymphocytes under static conditions. (Data not shown. Experiments were carried out by Susanna Fagerholm.) In addition, we have shown that effector cytotoxic T lymphocytes did not require PKC or Akt signalling and consequently not Rap1 for integrin-mediated adhesion under shear flow (Figure 45). It was also observed in Akt inhibition did not affect $CD4^+$ or $CD8^+$ T cell adhesion to ICAM-1 under shear flow (Figure 33) or when shear flow is absent (Figure 25). In contrast, Akt affects B cells in cell adhesion, indicating that adhesion is differently regulated in B cells and T cells.

Ro-31-8220 which inhibits classical and novel PKC did not reduce effector cytotoxic T lymphocyte adhesion under shear flow (Figure 45). However, future investigations using effector cytotoxic T lymphocytes with PKCζ inhibitors or cells lacking PKCζ could be carried out to assess if the PKCζ atypical isoform of PKC plays a role upstream of integrins in shear flow conditions or in cell-cell adhesion and migration in static conditions. Kinase profile of Syk II inhibitor documented the effectiveness of these inhibitors (Elcombe et al., 2013). Experiments to exclude the involvement of Syk could also be confirmed using knockout cells. In addition, assays which could measure calcium flux or calcium levels could be used to confirm the importance of calcium/calmodulin in regulation effector cytotoxic T cells in integrin-mediated cell adhesion, which can support the studies done using W-7 and calmidazolium.

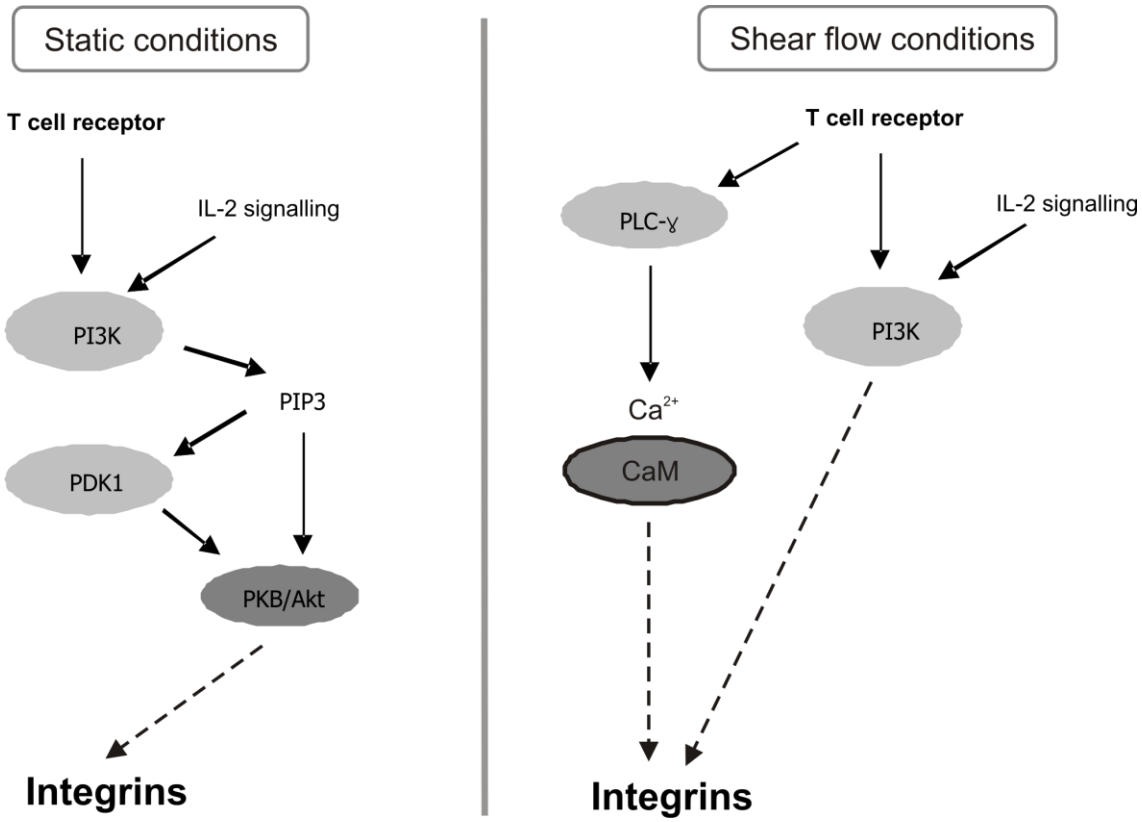


Figure 47. Schematic diagram comparing how T cell receptor and IL-2 signalling induced inside-out signalling can affect the activation of integrins in effector cytotoxic T lymphocytes under static and in shear flow conditions.

In static conditions, the PI3K/PDK1/Akt pathway was activated, and regulated integrin-mediated cell adhesion to ICAM-1 and cell migration. However, in shear flow conditions, although PI3K which is activated due to IL-2 signalling could also contribute to cell adhesion, calcium binding to Calmodulin downstream of the Phospholipase C pathway played a bigger role in regulating effector cytotoxic T lymphocyte adhesion. Calcium is ubiquitious for regulating and providing crosstalk for various cell signalling processes. The speed and ability to provide spatial and temporal control of calcium signalling has been suggested to control various processes such as integrin regulation, cell proliferation, transcription, motility and migration (Blytheston et al., 1999, Berridge et al., 2000). It can be hypothesised that effector cytotoxic T

lymphocytes use calcium/calmodulin signalling, together with a dynamic actin cytoskeleton, as a rapid mechanism to regulate integrin-mediated cell-adhesion under shear flow. Downstream effectors of calcium/calmodulin in integrin regulation in effector cytotoxic T lymphocytes remain to be determined, although there has been evidence suggesting that downstream of calmodulin, calcium-calmodulin-dependent protein kinase II (CaMKII), integrin cytoplasmic domain associated protein-1alpha (ICAP-1) and/or myosin light chain kinase (MLCK) may regulate the actin cytoskeleton and integrin-mediated adhesion (Bouvard and Block, 1998, Fagerholm et al., 2001).

However, the use of calmodulin antagonists such W-7, has been shown to be ineffective in inhibition of MLCK and CaMKII in T cells (Fagerholm et al., 2001). ICAP-1 has been suggested to be regulated by CaMKII and phosphorylation of ICAP-1 leads to the inhibition of αVβ1 integrins in CHO cells. ICAP-1 provides negative regulation of integrins by preventing talin association at the integrin cytoplasmic tail (Bouvard and Block, 1998, Bouvard et al., 2003). Whether this is applicable to T lymphocytes is not known at present.

Also, the actin cytoskeleton plays an important role in cell spreading, which enables cells to “grip” onto immobilized ICAM-1 to resist shear flow; it is possible that calcium/calmodulin regulates the actin cytoskeleton in cells for firm adhesion under shear flow conditions.

4.5. Conclusion

We have now established the conditions for carrying out physiologically relevant shear flow adhesion assays for effector cytotoxic T lymphocytes. The role of both the PKC and the PI 3-kinase/Akt axes in integrin regulation in different lymphocyte subtypes, and putative downstream effectors (such as Rap1) in lymphocyte adhesion and migration in static and shear flow conditions has also been investigated. It was shown that effector cytotoxic T lymphocytes do not require chemokine-induced or T cell receptor induced inside-out integrin signalling for cell capture or adhesion under shear flow. Inhibition of PKC or Akt also did not affect effector cytotoxic T lymphocyte adhesion to ICAM-1 under these conditions. These cells depend more on calcium/calmodulin and the ability of actin remodelling for cell adhesion to the endothelial cell layer under shear flow conditions. Despite difference in cell adhesion between lymphocyte subsets under shear stress, PI3K/Akt inhibition reduces cell adhesion in both B cells and effector cytotoxic T lymphocytes in static conditions.

5. Regulation of integrin recycling and phagocytosis

5.1. Introduction

5.1.1. Integrin turnover in Innate Immune cells

The ability to quickly recycle receptors like integrins is important and necessary for cell adhesion, polarization and tumour invasiveness. In cell migration, integrins and other membrane receptors are moved from the back to the front of the cell to provide the cells with a supply of integrins to form new adhesions. As the cell moves forward, integrins and adhesive material at the rear of cell (trailing uropod) are endocytosed and delivered to the front (Pierini et al., 2000). Recycling of β2-integrins required phosphorylation of myosin light chain and calcium signalling. Blocking these processes leads to cell arrest (Lawson and Maxfield, 1995, Eddy et al., 2000). About 30% of the surface LFA-1 was found to be internalised every 30-40 minutes in CHO (Chinese Hamster Ovarian) cells. The internalisation motif YxxØ (where x represents any amino acid and Ø represents amino acid with a bulky hydrophobic residue) is found in β2 and β7 integrins and this motif is also detected in transferrin receptors and other transmembrane proteins. (Fabbri et al., 1999).

After integrins have performed their tasks as adhesion and signalling receptors, they can be internalised and undergo degradation or recycling, like most other cell surface proteins. Trafficking can occur by various endo-exocytic pathways such as clathrin-dependent or caveolin-dependent pathways, rapid or slow recycling routes or via the trans-Golgi network or via the endoplasmic reticulum compartments to the plasma

membrane (Caswell and Norman, 2006, Caswell et al., 2009, Grant and Donaldson 2009).

However, integrin recycling in immune cells is not fully understood. In neutrophils, β2 integrin recycling is regulated via a cholesterol-sensitive clathrin-independent endocytic route during crawling on ICAM-1 surfaces (Fabbri et al., 2005), but the molecular details have not been extensively studied. Rab11, a small GTPase that regulates Rac activity in actin dynamics responsible for the formation of cell protrusions in cell migration and cell-cell communications, is also important in recycling of proteins (Takahashi et al., 2012, Ramel et al. 2013). Rab11 mutants have been found to affect neutrophil migration by causing integrin accumulating after endocytosis and by preventing integrins from aggregating at the leading edge of cells in the presence of chemoattractants (Barreiro et al., 2007). Mutations in the cytoplasmic tail of CR3 (also known as Mac-1) have also been suggested to compromise phagocytosis and integrin recycling but recycling of integrins during phagocytosis had not been investigated in detail (Wiedermann et al., 2006).

5.1.2. TTT/AAA-β2-integrin Knock-in mouse model

The Thr758-760 motif in the cytoplasmic domain of the β2 integrin is known to regulate LFA-1 integrin-mediated adhesion. Recently, Kindlin-3 was found to bind to this site in β2 integrins in transfected HEK293 cells and it was demonstrated that the interaction of Kindlin-3 and the β2 integrin in T cells was necessary for cell adhesion and homing (Morrison et al., 2013). Patients suffering from Leukocyte Adhesion Deficiency (Type I) have mutations which lead to deficiency or absence of β2 integrin which affected firm

adhesion of leukocytes. Patients with Leukocyte Adhesion Deficiency (Type III) have defective inside-out integrin signalling due to kindlin-3 mutations affecting kindlin expression or integrin binding (Harris et al., 2013). Neutrophils from LAD I patients were found to be deficient in LFA-1 and were unable to stimulate phagocytosis (Gresham et al., 1991). A β2-integrin knock-out mouse model suffered from recurring muco-cutaneous infections and chronic dermatitis with spontaneous skin ulceration, amongst other symptoms (Marski et al 2005), while mice lacking kindlin-3 die shortly after birth from severe bleeding (Moser et al., 2008, Moser et al., 2009). A mouse model where the TTT motif in the β2 integrin cytoplasmic domain was mutated to AAA is available in our laboratory (made by Taconic Artemis), termed the "TTT/AAA knock-in" mouse line in this chapter. These mice appeared healthy, unlike the kindlin-3 knock-out mice described in previous publications (Morrison et al, 2013).

5.1.3. Regulatory proteins that may potentially bind at T758-760 in the β2 integrin

Activation of β2 integrins have been shown to be important in regulation of integrin function in lymphocytes. Phosphorylation at T758 leads to the recruitment of 14-3-3 adaptor proteins to the β2 integrin cytoplasmic domain which in turn activates actin cytoskeleton modulators that are involved in cell spreading, such as Rac1/Cdc42 in cell lines and in primary T cells (Fagerholm et al, 2002, Fagerholm et al, 2005, Nurmi et al, 2007, Gronholm et al, 2011). T758 phosphorylation also negatively regulates filamin recruitment to the integrin; filamin is a negative regulator of integrin-mediated cell adhesion (Takala et al., 2008). Kindlins are focal adhesion proteins which have a similar structure as another focal adhesion protein, talin as they both contain FERM domain

structures. Kindlin-3 is exclusively expressed in haematopoietic cells while kindlin-2 is ubiquitous in all cell types (Moser et al., 2009).

Kindlin-2 is necessary for β1-integrin activation in fibroblasts and binds to a site which is very similar to the Thr758-760 site in the cytoplasmic tail of the β2 integrin where kindlin-3 binding occurs (Figure 48, Yates et al., 2012). Another protein that shares the kindlin binding site in the β1-integrin is Sorting Nexin-17 (nexin-17), which is found in endosomes. Binding of nexin-17 to the β1 integrin cytoplasmic tail in endosomes after endocytosis prevented the lysosomal degradation of β1 integrins (Böttcher et al., 2012). Sorting nexins are a family of trafficking proteins with a PX-domain (phospholipid-binding motif) which is responsible for the transport of membrane receptors in the endocytic pathway (Worby and Dixon, 2002). Another example of a sorting nexin is sorting nexin-4 (SNX4) which interacts with the transferrin receptor in the early endosomes and endosomal recycling compartment near the cell nucleus to divert proteins away from lysosomal degradation in HeLa cells (Traer et al., 2007).

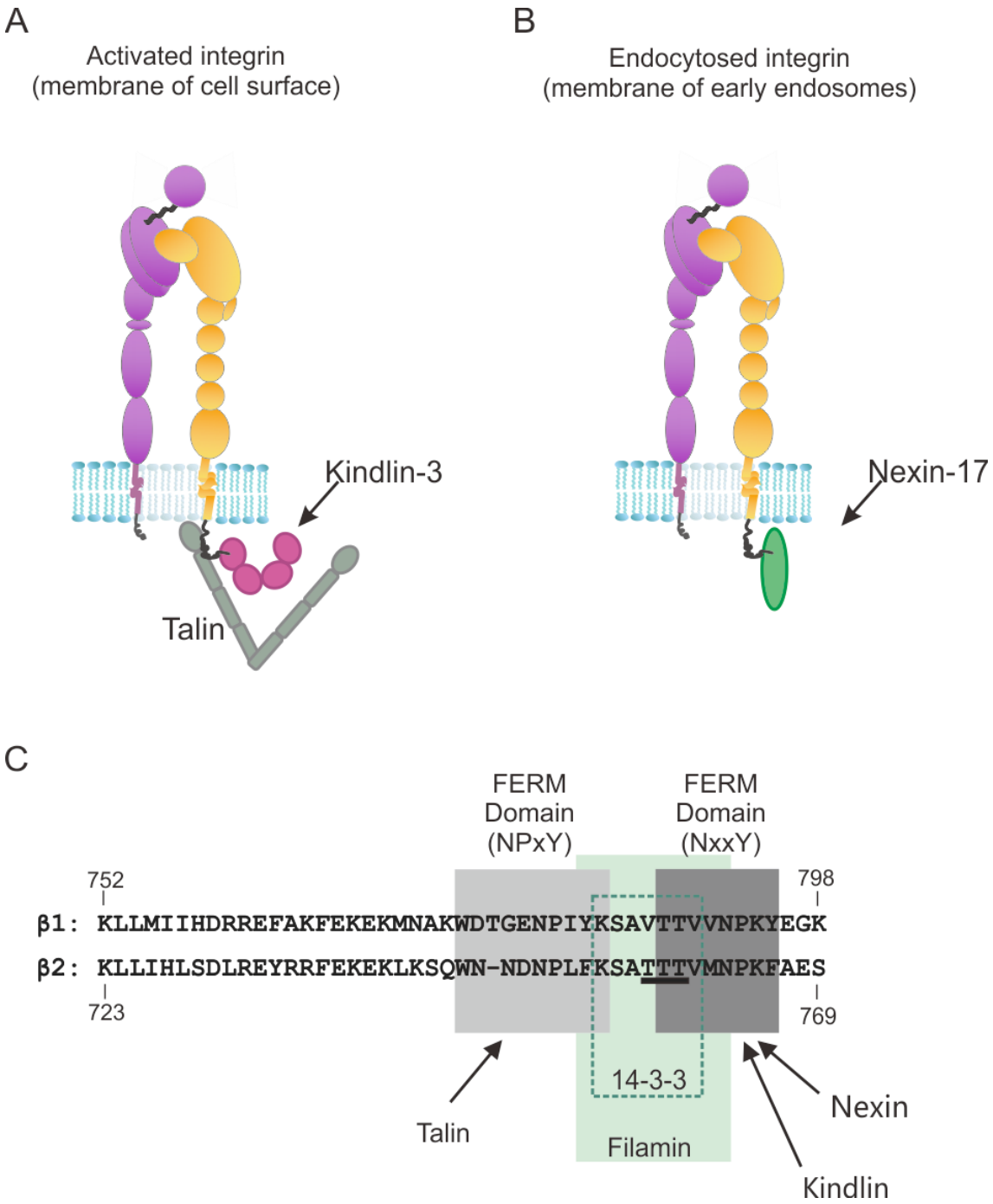


Figure 48. Sequence alignment of human β1 and β2 integrin sequences.

(A) Structure of integrin at its highly activated state with talin and kindlin-3 binding to β2-integrin, similar to when kindlin-2 binds to β1-integrin. (B) Structure of integrin after activation and where sorting nexin-17 is thought to bind to the cytoplasmic domain of the β2-integrin tail when kindlin-3 is detached (or when kindlin-2 is detached from β1-integrin). (C) Mouse β1 and β2 integrin sequences were aligned at the FERM domains where kindlin (dark grey box where NxxY is located) and talin (light grey box where NPxY is located) bind to during integrin

activation (Yates et al., 2012). This kindlin binding site overlaps with the nexin-17 binding site in β1 integrins. The important TTT phosphorylation motif (758-760) in the β2 integrin is underlined. The binding site where 14-3-3 binds to when the TTT motif is phosphorylated is marked with a box outlined by dark green dotted line (Gahmberg et al., 2009). Filamin, a negative regulator of integrin activation binds at the NPxY motif when this site is unphosphorylated iss shown in the green area which overlaps with the 2 FERM domains needed for Talin and Kindlin binding (Kiema et al., 2005).

Significant reduction in kindlin-3 binding to TTT/AAA mutated β2 integrins has been reported by our group (Morrison et al., 2013). The TTT/AAA knock-in mice available in the laboratory are ideal for investigations of the putative role of the TTT-motif/nexin in recycling of β2 integrins in leukocytes, as well as studies of integrin-mediated adhesion and phagocytosis.

5.1.4. The rs1143679 (R77H) systemic lupus erythematosus variant of ITGAM (αM integrin)

Recently, it has been reported that an rs1143679 variant of the ITGAM gene, which encodes for the αM integrin, is associated with systemic lupus erythematosus. This genetic alteration leads to an R77H mutation in the αM integrin extracellular domain (Figure 49, MacPherson et al., 2011). Systemic lupus erythematosus patients often have overactive B cell production of autoantibodies, vasculitis, reduced immune tolerance and tissue injury, which is classified under Type III Hypersensitivity often linked with lack of immune clearance capabilities (Harley et al., 2008, Nath et al., 2008, Macpherson et al., 2013). Mac-1 regulates cell adhesion but also negatively regulates TLR signalling and Th17 cell differentiation and inhibits T cell activation (Fagerholm et

al., 2013). The Mac-1 integrin is also known as CR3 and binds to iC3b during complement-mediated phagocytosis. How the R77H mutation in αM integrins affects Mac-1–mediated functions such as adhesion and phagocytosis was not clear at this point.

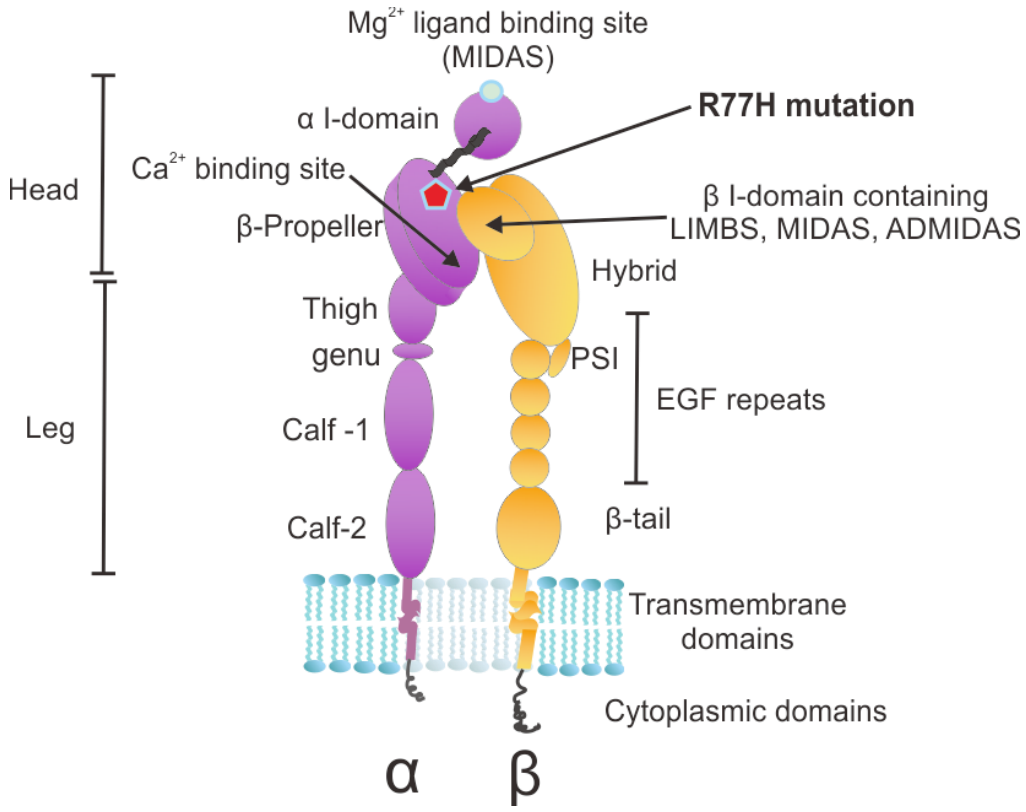


Figure 49. R77H mutation in the Mac-1 integrin

Location of R77H mutation found in αM integrin is marked by a red hexagon. Figure 49 is adapted from Figure 2.

5.2. Aims of this Chapter

The aim of this chapter was to investigate β2 integrin regulation in myeloid cells, particularly the role of the TTT-domain in the β2 integrin. In myeloid cells, integrins are important in the regulation of cell adhesion and migration as well as in phagocytosis of complement-opsonised particles. Whether the TTT-domain regulates these processes in β2 integrins was unclear. Also, the putative effect of the TTT/AAA mutation on integrin recycling would be investigated. As it was unclear how the systemic lupus erythematosus-associated R77H mutation in the αM integrin affected integrin-mediated processes such as cell adhesion and phagocytosis at the molecular or cellular level, this would also be investigated. Taken together, we hoped to achieve a better understanding of the regulation of integrin-mediated processes in macrophages and dendritic cells relating to immunodeficiency, e.g., Leukocyte Adhesion Deficiency, and hypersensitivity, e.g., Systemic Lupus Erythematosus.

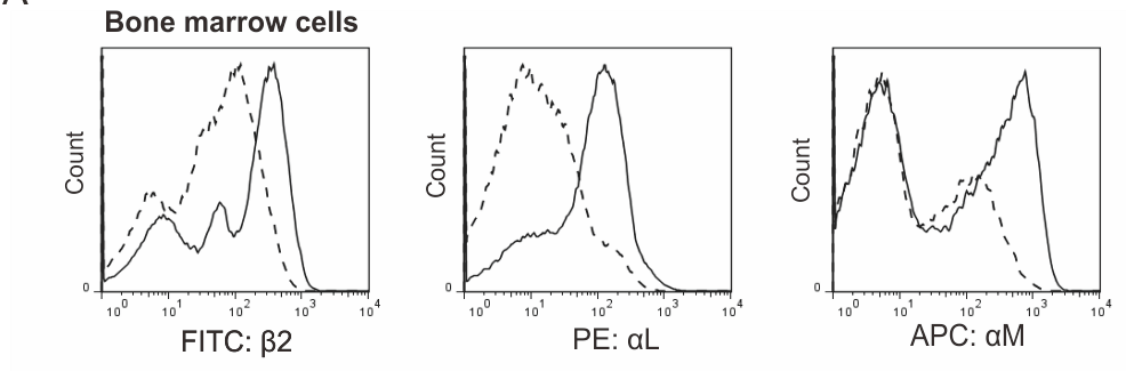
5.3. Results

5.3.1. Integrin surface expression in various primary immune cell populations

Cell surface expression of integrins was lower in bone marrow and spleen cells from TTT/AAA knock-in mice compared to wild-type mice.

Firstly, it was important to investigate β2 integrin expression in immune cell populations. It was also important to assess if the TTT/AAA mutation had an effect on surface expression of β2 integrins. We used flow cytometry analysis to examine surface expression of the integrin β2 subunit, and the integrin α subunits (αL and αM). We analysed immune cells from bone marrow, spleen (consisting ~55% of B cells as shown by Pellegrini et al., 2007) and effector cytotoxic T lymphocytes established from spleens.

A



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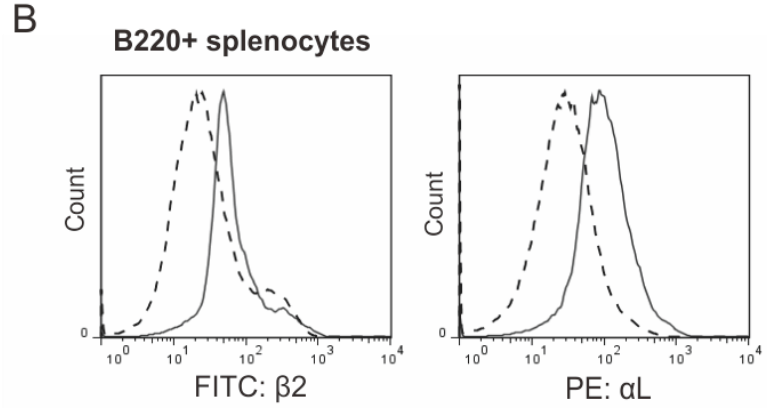

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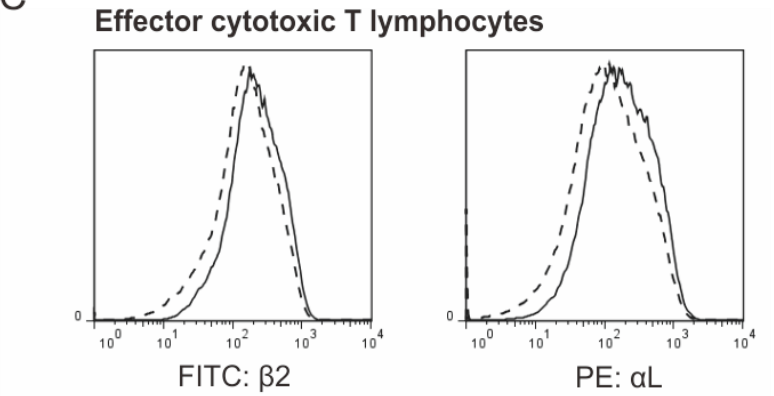


D

MFI Readings

	β2	αL	αM
WT (Bone Marrow)	273	17.4	3.7
KI (Bone Marrow)	130	3.0	1.8
WT (Splenocytes)	39.8	57.7	
KI (Splenocytes)	25.3	29.8	
WT(CTL)	392	266	
KI (CTL)	213	110	

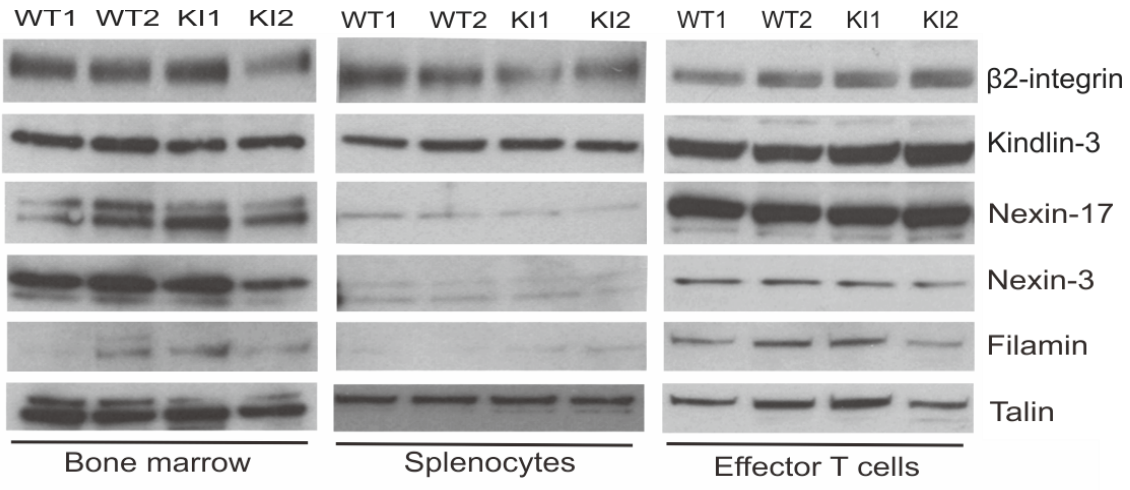
Figure 50. The TTT-motif affects surface expression of integrins in certain immune cell populations.

Integrin surface expression in primary leukocyte populations from wild-type and TTT/AAA knock-in mice were compared using flow cytometry. Fluorescently labelled antibodies were used to detect β2, αL and αM integrins. Cells were gated to a live population, and to B220 positive cells (to detect B cells) in the splenocyte population. (A) Integrin expression in cells from bone marrow, (B) spleen cells and (C) effector cytotoxic T lymphocytes. In all figures solid lines represent readings from wild-type mice and dashed lines represent readings from TTT/AAA knock-in mice. (D) MFI (median fluorescence intensity) readings from above flow cytometry results. Results are representative of N=2 mice.

Wild-type bone marrow cells expressed high levels of β2, αL and αM integrins on the cell surface. Cells from TTT/AAA knock-in mice also had high levels of cell surface β2 integrins but the expression was lower than that from wild-type mice. The expression of the αL integrin in TTT/AAA knock-in cells was much lower compared to that in wild-type cells. However, in both wild-type and knock-in bone marrow cells there were 2 distinct population of cells with different αM integrin expression, with a significant population of cells that had low αM integrin levels. More than half of the wild-type cells expressed high levels of the αM integrin while a much smaller population of TTT/AAA knock-in cells expressed αM integrins (Figure 50A). $B220^{+}$ spleen cells from wild-type mice expressed more β2 and αL integrins on the cell surface than TTT/AAA knock-in mouse spleen cells (Figure 50B). In contrast, effector cytotoxic T lymphocytes from TTT/AAA knock-in mice expressed only slightly lower levels of β2 and αL integrins compared to effector cytotoxic T lymphocytes from wild-type mice (Figure 50C). αM integrins were not present in splenocytes and effector cytotoxic T lymphocytes. These results showed that there was a difference in surface integrin expression in cells from bone marrow and spleens between wild-type and TTT/AAA-β2-integrin KI mice, but this difference was much smaller in effector cytotoxic T lymphocytes. The differences

in expression of integrins in these immune cell populations indicated that integrins were differently regulated.

Surface expression of integrins expressed does not reflect the total amount of integrins present in cells. Therefore, western blot detection of integrins and other cytoplasmic proteins in whole cell lysates of the various immune cell populations was carried out.



26474	19688	25869	18914	40275	25706	21112	31042	8219	10022	9318	9057
11880	20799	24097	18628	5414	3411	3461	4001	22576	21862	19426	19005
25972	23226	25343	17250	12716	8993	11239	9484	5106	4624	3294	2146
7305	13635	15858	12802	6164	2832	4920	7498	5664	7996	7343	3139
24345	17923	19949	18694	29618	24475	26792	27678	5825	7753	7513	4118

β2-integrin
Nexin-17
Nexin-3
Filamin
Talin

Densitometry Readings adjusted to Kindlin-3 levels

Figure 51. Western blot detection of β2 integrins and cytoplasmic proteins in cell lysates from different immune cell populations.

Whole cell lysates were prepared from cells from bone marrow, spleens and effector cytotoxic T lymphocytes from wild-type and TTT/AAA knock-in mice. β2 integrins and potential interacting cytoplasmic proteins; kindlin-3, nexin-17, nexin-3, filamin and talin were detected using western blot techniques. Data representative of N=2 experiments, 4 pairs of mice.

Total β2 integrin levels in cells from bone marrow, splenocytes and effector cytotoxic T lymphocytes from wild-type and TTT/AAA knock-in mice were similar despite a clear difference in cell surface levels of β2 integrin levels detected by flow cytometry in splenocytes and bone marrow cells (Figure 50A and 51). There was also no clear difference in levels of cytoplasmic integrin-interacting proteins such as kindlin-3, nexin-17, nexin-3, filamin or talin in cells from bone marrow, splenocytes and effector cytotoxic T lymphocytes between wild-type mice and TTT/AAA knock-in mice. Yeast II hybrid screening using the αM integrin as bait had suggested that nexin-3 was a possible binding target of αM integrins and therefore it was included in this study. (Experiment carried out by Matthew MacPherson. Data not shown.)

5.3.2. Regulation of integrins in bone marrow-derived dendritic cells and macrophages.

Cell surface expression of integrins was lower in bone marrow-derived dendritic cells and macrophages from TTT/AAA knock-in mice than in cells from wild-type mice.

The bone marrow contains progenitor cells for dendritic cells and macrophages. Bone marrow cells had high levels of β2 integrins and the TTT-mutation affected surface expression in these cells. Therefore, we investigated if mature dendritic cells and macrophages also had high levels of β2 integrins, and the effect of the TTT-mutation on integrin expression in these cells.

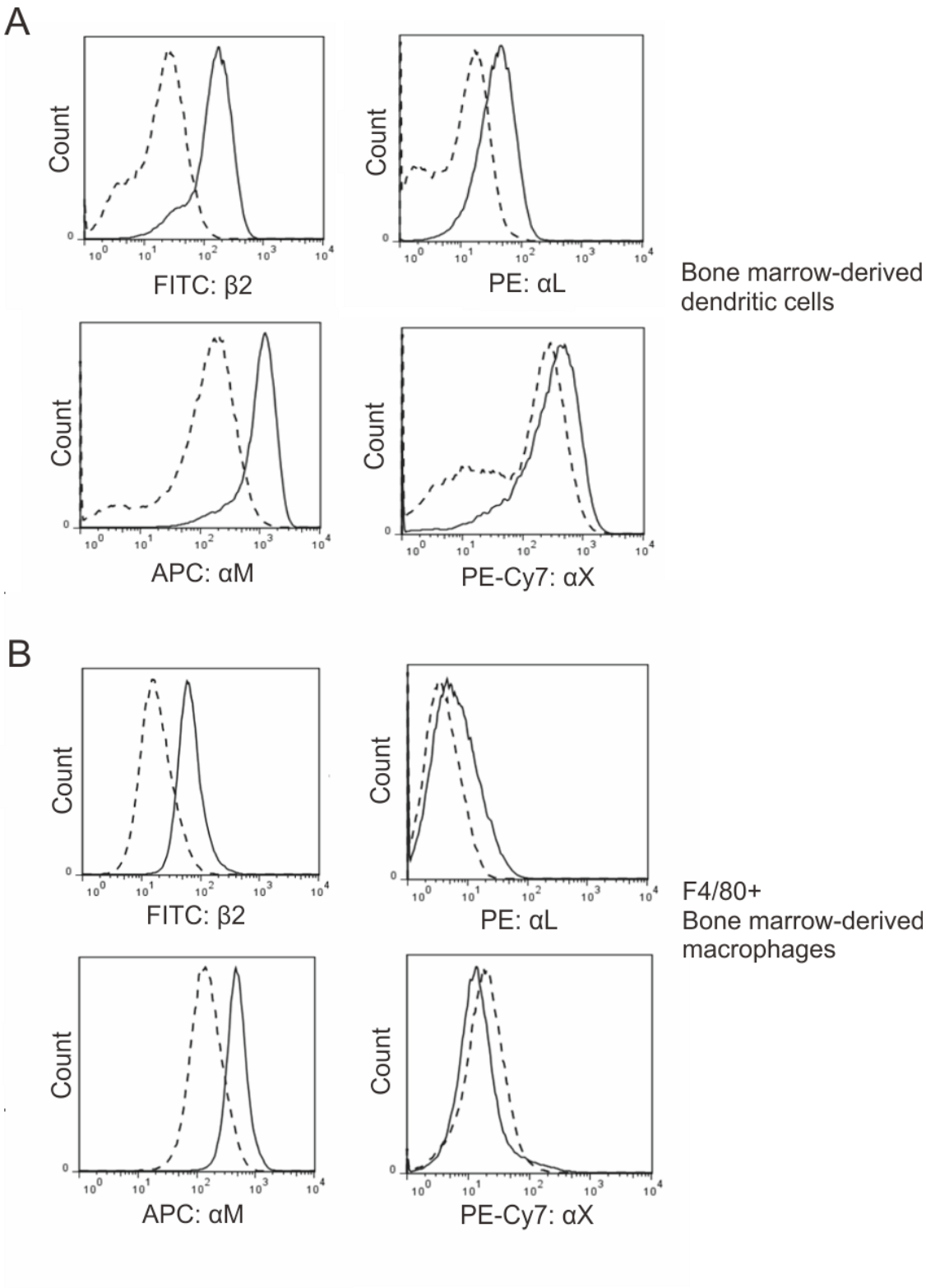
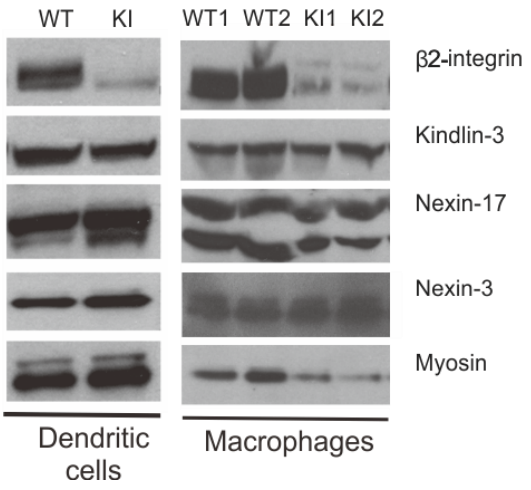


Figure 52. Expression of integrins in bone marrow-derived dendritic cells and macrophages from wild-type and TTT/AAA mice.

Fluorescently labelled antibodies were used to detect β2, αL, αM and αX integrins on cell surfaces. Cells were gated to a live population for dendritic cells and additionally gated for

F4/80 positive cells for the macrophage population. Flow cytometry analysis was used to detect surface expression of integrins in (A) dendritic cells and (B) macrophages. In all figures solid lines represent readings from wild-type mice and dashed lines represent readings from TTT/AAA knock-in mice. . Results are representative 2 experiments, N=4 pairs of mice.

Bone marrow-derived dendritic cells from wild-type mice had high levels of β2, αL, αM and αX integrins on the cell surface. The high level of αX in dendritic cells was expected as positive expression of αX (also known as CD11c) is traditionally used to identify and gate the dendritic cell population. Cells from TTT/AAA knock-in mice had much lower levels β2, αL and αM integrins than wild-type cells. In addition, although there was a population of cells that expressed similar levels of αX as wild-type cells, there was also a population of cells that had much lower levels of αX (Figure 52A). Bone marrow derived macrophages that were positive for F4/80 (a marker for identifying the macrophage population) from wild-type mice had high levels of β2- and αM integrins but low expression of the αL-chain. Macrophages from TTT/AAA knock-in mice had lower cell surface expression of β2- and αM integrins compared to wild-type macrophages and also very low expression of αL-chains like wild-type macrophages (Figure 52B). Both wild-type and TTT/AAA knock-in macrophages had low levels of the αX-integrin when compared to dendritic cells (Figure 52A and 52B).



Densitometry Readings
(Adjusted to Kindlin-3 as control)

	WT	KI	WT1	WT2	KI1	KI2
β2-integrin	23696	7230	29618	25940	10148	5580
Kindlin-3						
Nexin-17	24178	32970	39176	26380	31992	39591
Nexin-3	10882	15924	27098	18893	30504	36693
Myosin	22255	28513	8991	10114	6783	4257

Figure 53. Western blot detection of β2 integrins and cytoplasmic proteins in cell lysates from bone marrow-derived dendritic cells and macrophages.

Whole cell lysates were obtained from dendritic cells and macrophages derived from the bone marrow of wild-type and TTT/AAA knock-in mice. β2 integrins and potential interacting cytoplasmic proteins; kindlin-3, nexin-17 and nexin-3 were detected using western blot techniques, while kindlin-3 were used as controls instead of myosin due to variation of myosin levels between cell types. Densitometry readings corrected to kindlin-3 used as controls are presented in the table next to the blot. N=2 pairs of mice.

Western blot detection of β2 integrins and cytoplasmic proteins (kindlin-3, nexin-17, nexin-3 and myosin) was carried out using lysates obtained from bone marrow-derived dendritic cells and macrophages. Kindlin-3 were used as loading controls instead of myosin as myosin levels varied between wild-type and TTT/AAA knock-in cells and could not be detected some immune cell types (such as B cells and bone marrow cells, data not shown). TTT/AAA knock-in bone marrow derived dendritic cells and macrophages had significantly less β2 integrins than wild-type dendritic cells but the

levels of nexin-17 and nexin-3 were similar in both wild-type cells and TTT/AAA knock-in cells after normalisation to kindlin-3 as control (Figure 53).

In summary, the results of flow cytometry and western blot experiments showed that the TTT/AAA mutation leads to lower levels of αLβ2 (LFA-1) and αMβ2 (Mac-1) in bone marrow-derived dendritic cells and lower levels of Mac-1 in bone marrow- derived macrophages. The difference in levels of surface expression of integrins in wild-type and TTT/AAA knock-in dendritic cells and macrophages could be due to problems in integrin recycling (Figure 52 and 53).

5.3.3. Involvement of the TTT-motif in β2 integrin recycling and adhesion.

After integrin-mediated cell adhesion and signalling, kindlin-2 disassociates from the integrin cytoplasmic tail TT-motif. Nexin-17 binding to the TT-motif in the integrin in endosomes protects integrins from lysosomal degradation (Böttcher et al., 2012). Reduction in kindlin-3 binding to the alanine mutated T758-760 site in β2 integrins has been reported by our group (Morrison et al., 2013). Similarly, we hypothesized that nexin (nexin-17 or nexin-3) would bind to the Thr758-760 site in the β2 integrin cytoplasmic tail during the internalisation of integrins to prevent lysosomal degradation of integrins during integrin recycling.

Rescue of integrin surface expression in dendritic cells using inhibitors blocking different stages of the endocytic pathway.

To investigate if the Thr758-760 site in the β2 integrin tail protects integrins from being degraded after internalisation, thus affecting integrin surface expression, bone marrow derived dendritic cells were left untreated or pre-treated with a proteasome inhibitor (MG-132), a lysosome inhibitor (bafilomycin) or a late endosome inhibitor (primaquine) to inhibit protein degradation. Integrins on cell surface were then detected using flow cytometry. MG-132 is a proteasome inhibitor which binds to the 26S subunit of the proteasome, thereby inhibiting its capability to degrade ubiquitin-tagged proteins (Han and Park, 2002, Goldberg, 2012). Bafilomycin works by inhibiting vacuolar-type H+-ATPases needed for the acidification in lysosomes (Yoshimori et al., 1991). Primaquine was found to interfere with membrane receptor recycling by interfering with Calmodulin, thereby affecting transport from endosomes to the plasma membrane. Primaquine could also neutralise the vacuolar pH in endosomes. Bafilomycin was also found to reverse the effects of primaquine (van Weert et al., 2000).

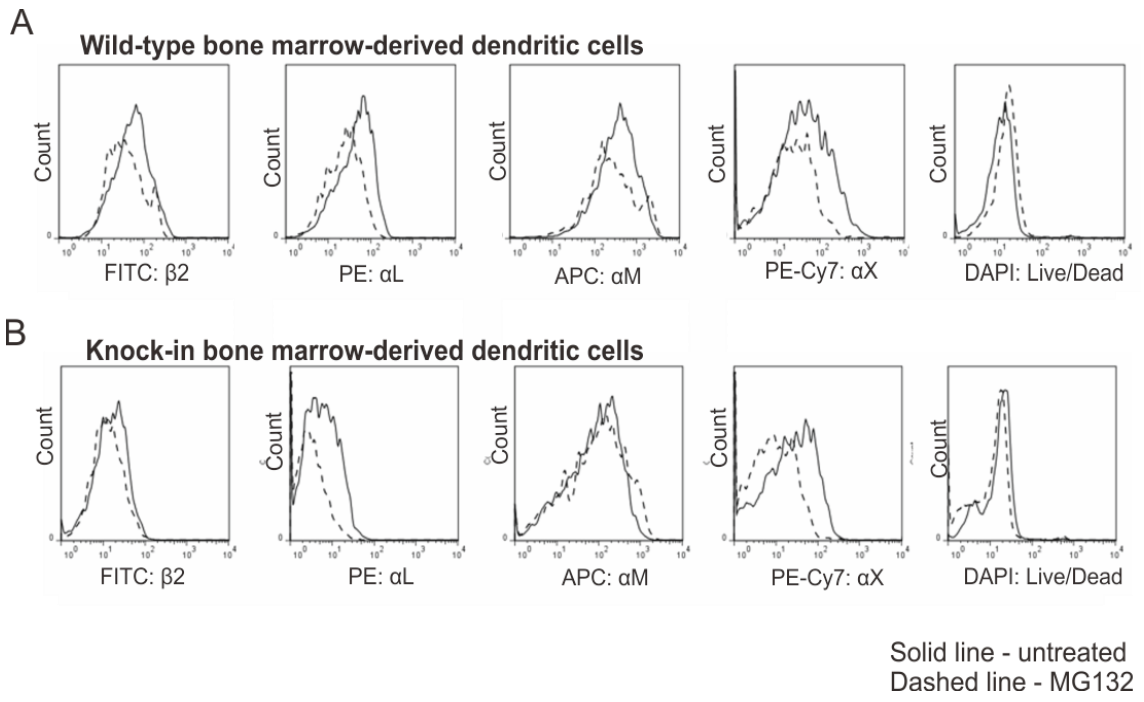


Figure 54. Treatment of TTT/AAA knock-in bone marrow-derived dendritic cells with the proteasome inhibitor MG-132 did not result in rescue of integrin surface expression.

Levels of cell-surface β2, αL, αM and αX integrins on (A) wild-type dendritic cells or (B) knock-in dendritic cells were compared by flow cytometry after 1 uM MG-132 treatment overnight. The solid line represents untreated cells while the dashed line represents MG-132-treated cells. Cells were gated to a live population using DAPI-stain and the live/dead population is shown in the last column. Results are representative of N=3 experiments, 6 pairs of mice.

As shown previously, cell surface expression of integrins was lower in bone marrow-derived dendritic cells and macrophages from TTT/AAA knock-in mice compared to wild-type mice (Figure 51). Treatment of bone marrow-derived dendritic cells from wild-type mice and TTT/AAA knock-in mice with the proteasome inhibitor MG-132 did not improve levels of β2, αL, αM and αX integrin surface expression and appeared to even reduce these levels further in wild-type cells. MG-132 used at 1μM did not affect cell viability as the live/dead stain showed that cell death was limited in the

presence of the inhibitor. Taken together, the proteasome inhibitor MG-132 did not rescue cell surface integrin levels in TTT/AAA-β2 integrin expressing cells by preventing proteasome degradation of integrins, indicating that the proteasome pathway was not involved in regulating the surface expression level of integrins in these cells (Figure 54).

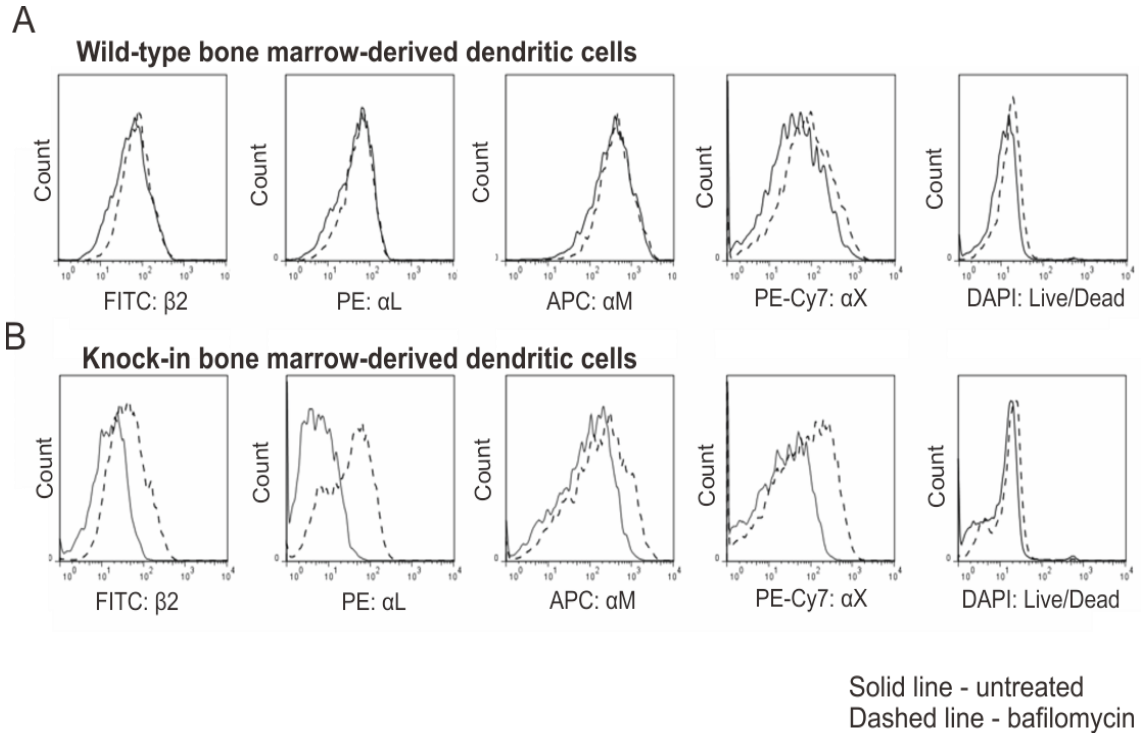


Figure 55. Treatment of TTT/AAA knock-in mice bone marrow-derived dendritic cells with the lysosome inhibitor bafilomycin resulted in the rescue of cell surface integrins.

Levels of cell-surface β2, αL, αM and αX integrins on (A) wild-type dendritic cells or (B) knock-in dendritic cells were compared with and without 12.5 nM bafilomycin treatment for 4hours. Solid lines represent untreated cells while dashed lines represent bafilomycin-treated cells. Cells were gated to a live population using DAPI-stain for integrin levels detection and the live/dead population is shown in the last column. Results are representative of N=3 experiments, 6 pairs of mice.

Treatment of bone marrow-derived dendritic cells from wild-type mice with a lysosome inhibitor, bafilomycin did not significantly affect β2, αL, αM and αX integrins in wild-type cells (Figure 55A). However, the inhibitor had a significant effect in TTT/AAA knock-in cells, resulting in increased cell surface levels of β2, αL, αM and αX integrin chains on dendritic cells, and bringing the expression up to the levels detected in wild-type dendritic cells (Figure 55A and 55B). αM cell surface levels were already high in TTT/AAA knock-in cells when compared to wild-type cells but bafilomycin could still increase the levels of cell surface αM integrin. Bafilomycin used at 12.5 nM was suitable as the live/dead stain showed that cell death was limited in the presence of the inhibitor. Therefore, the lysosome inhibitor bafilomycin rescued cell surface integrin levels in TTT/AAA knock-in dendritic cells to that on wild-type cells by preventing integrin degradation in lysosomes (Figure 55).

Compared with Figure 54, bone marrow-derived dendritic cells from TTT/AAA knock-in mice, pre-treated with proteasome inhibitor MG-132 did not rescue β2, αL, αM and αX integrins. Together with the results from Figure 54, these results showed that integrins that are not recycled back to the membrane surface are degraded by lysosomes instead of proteasomes and also suggested that unlike wild-type β2 integrins, TTT/AAA knock-in β2 integrins were more readily degraded in lysosomes instead of being recycled back to the membrane surface. These results also led to the suggestion that the TTT-motif in β2 integrins had the ability to protect integrins from lysosomal degradation, possibly through interaction with nexin-17, similarly to the reported observation that β1-integrins were protected from lysosomal degradation through interaction with this protein (Böttcher et al., 2012).

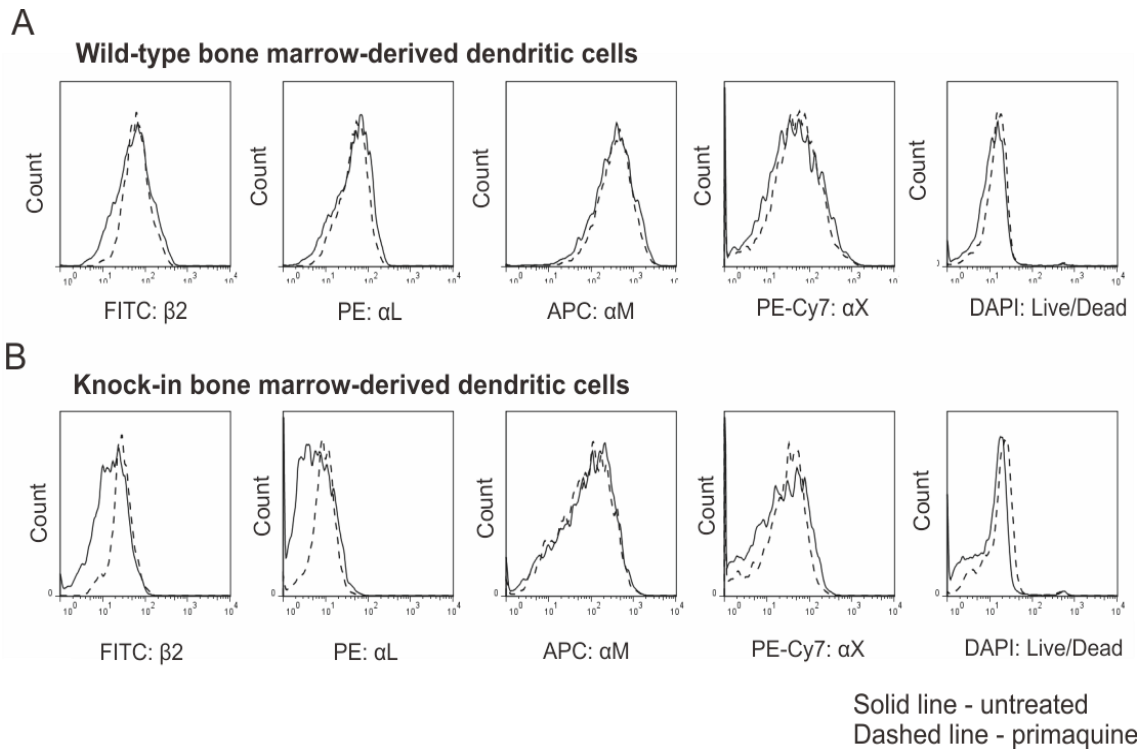


Figure 56. Treatment of TTT/AAA knock-in mice bone marrow-derived dendritic cells with the endosome inhibitor primaquine did not rescue cell surface integrins.

Levels of cell-surface β2, αL, αM and αX integrins on (A) wild-type dendritic cells or (B) knock-in dendritic cells were compared with and without 0.5 mM primaquine treatment for 20 minutes. Solid lines represent untreated cells while dashed lines represent bafilomycin-treated cells. Cells were gated to a live population using DAPI-stain for integrin levels detection and the live/dead population is shown in the last column. Results are representative of N=3 experiments, 6 pairs of mice.

Wild-type and TTT/AAA knock-in bone marrow-derived dendritic cells were also pretreated with endosome inhibitor primaquine. Primaquine enriches endogenous proteins by blocking the recycling of proteins in endosomes, affecting the transport from endosomes to the plasma membrane via transferrin, and neutralising vacuolar pH in early endosomes to prevent degradation of proteins (van Weert et al., 2000). There

was only some rescue of surface β2 and αL integrins which was not significant (Figure 56).

Rescue of integrin surface expression by inhibition of integrin lysosomal degradation does not rescue cell adhesion.

Since it was possible to rescue integrin levels in TTT/AAA knock-in bone marrow derived dendritic cells by treatment of cells with bafilomycin, we decided to investigate whether this increase in cell surface expression of TTT/AAA integrins would affect dendritic cell adhesion. A static adhesion assay using the β2-integrin ligand ICAM-1 was carried out to determine if this would occur.

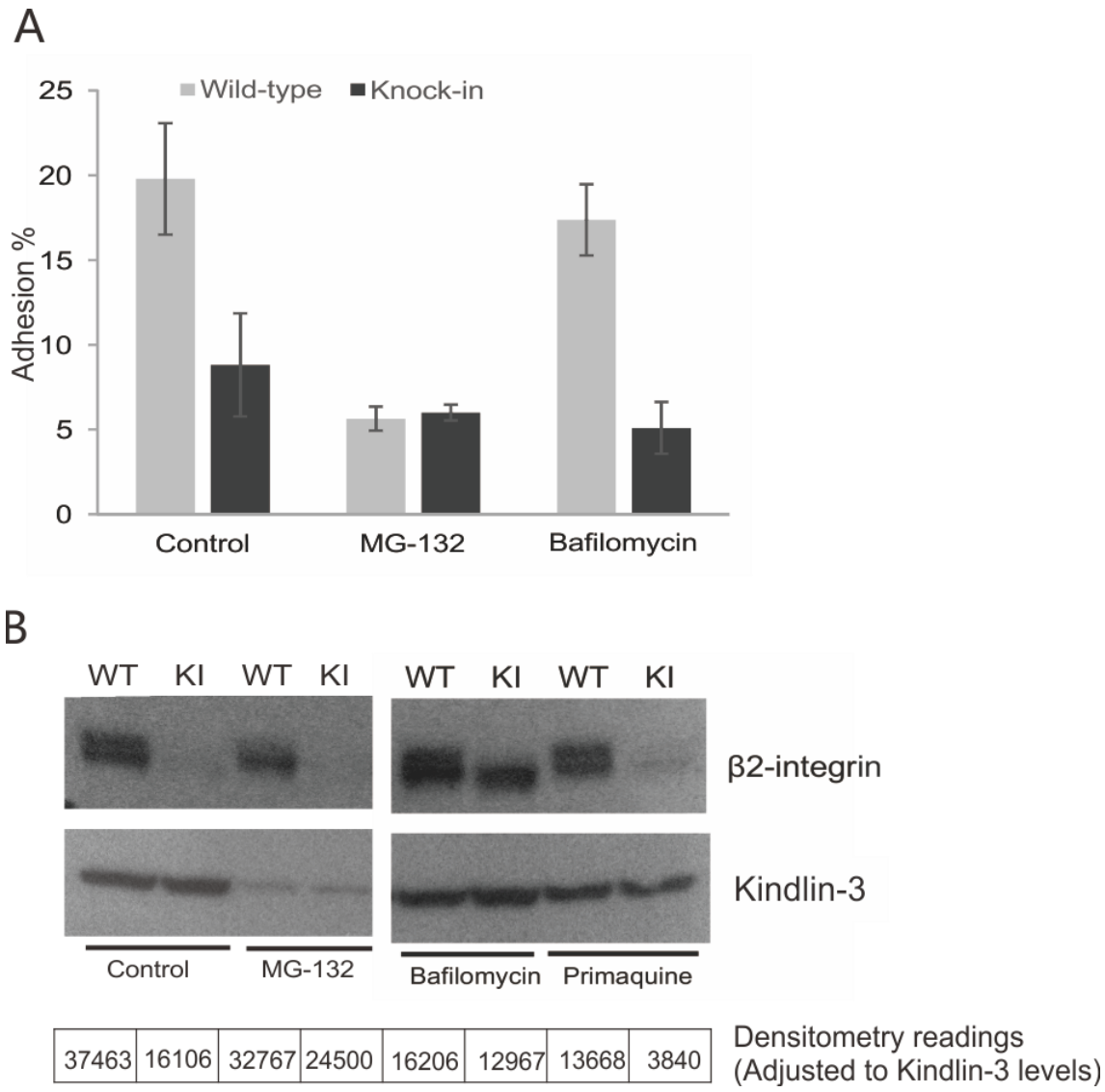


Figure 57. Static adhesion assay comparing the effects of proteasome and lysosome inhibitors on integrin-mediated adhesion of bone marrow-derived dendritic cells to ICAM-1.

(A) Bone marrow derived dendritic cells from wild-type and TTT/AAA knock-in mice were pre-treated with inhibitors (1uM MG-132 for 20 hours and 12.5nM bafilomycin for 4 hours), and static adhesion assays was carried out. Error bars represent S.D. (B) Western blot detection of β2 integrins and kindlin-3 from whole cell lysates before and after drug treatment (1uM MG-132 for 20hours, 12.5nM bafilomycin for 4hours and 0.5mM primaquine for 20minutes). Densitometry readings were adjusted to kindlin-3 levels as controls. N=2 l experiments, 4 pairs of mice.

Bone marrow derived dendritic cells from TTT/AAA knock-in mice show much lower adhesion to ICAM-1 compared to wild-type cells, confirming that this motif in the β2-integrin chain is important for cell adhesion in primary cells (N=2, p=0.075) (Figure 8A). Wild-type dendritic cells pre-treated with MG-132 had much lower cell adhesion to ICAM-1 than untreated cells even though cell death was limited (Figure 6, 7 and 8A).

TTT/AAA knock-in dendritic cells pre-treated with bafilomycin did not display improved cell adhesion to ICAM-1 and adhesion of these cells was significantly lower than wild-type cells with the same treatment of bafilomycin (N=2. $p<0.05$, Student's t-test) (Figure 57A). TTT/AAA knock-in bone marrow dendritic cells have less β2 integrins than wild-type cells detected on cell surface (Figure 54 and 55). TTT/AAA knock-in bone marrow dendritic cells pre-treated with bafilomycin showed significant increase in β2 integrins levels compared to cell lysate from untreated knock-in cells or cells pre-treated with MG-132 or primaquine (Figure 57B). All the cells had the same amount of Kindlin-3 levels except for MG-132 proteosome inhibitor treated cells, which displayed significantly reduced kindlin-3 levels, which could possibly also affect their cell adhesion properties (Figure 57). It was also noted that MG-132 treated cells looked slightly smaller after treatment but FACS analysis showed there was no obvious cell death (Data not shown and Figure 54). The proteasome inhibitor could affect other cell signalling pathways and degradation of other membrane proteins which could have had other indirect effect on kindlin-3 expression or cell adhesion that was not further investigated here (Figure 57B).

Taken together, the results from static adhesion assays and flow cytometry experiments suggested that although bafilomycin treatment rescued the cell surface β2-integrins on bone marrow derived dendritic cells, the integrins were not able to mediate cell

adhesion to ICAM-1. The explanation for this is probably because the Thr758-760 site is required for kindlin-3 to bind to the cytoplasmic tail of β2 integrins, which is in turn required for cell adhesion to take place.

Comparing the effects of bafilomycin in the rescue of cell surface integrin expression in dendritic cells and macrophages.

Bone marrow derived macrophages express high amounts of Mac-1 integrins and lower LFA-1 integrin amounts compared to bone marrow derived dendritic cells (Figure 51). Hence it was interesting to investigate how bafilomycin treatment would affect integrin expression in macrophages and how this compared to dendritic cells.

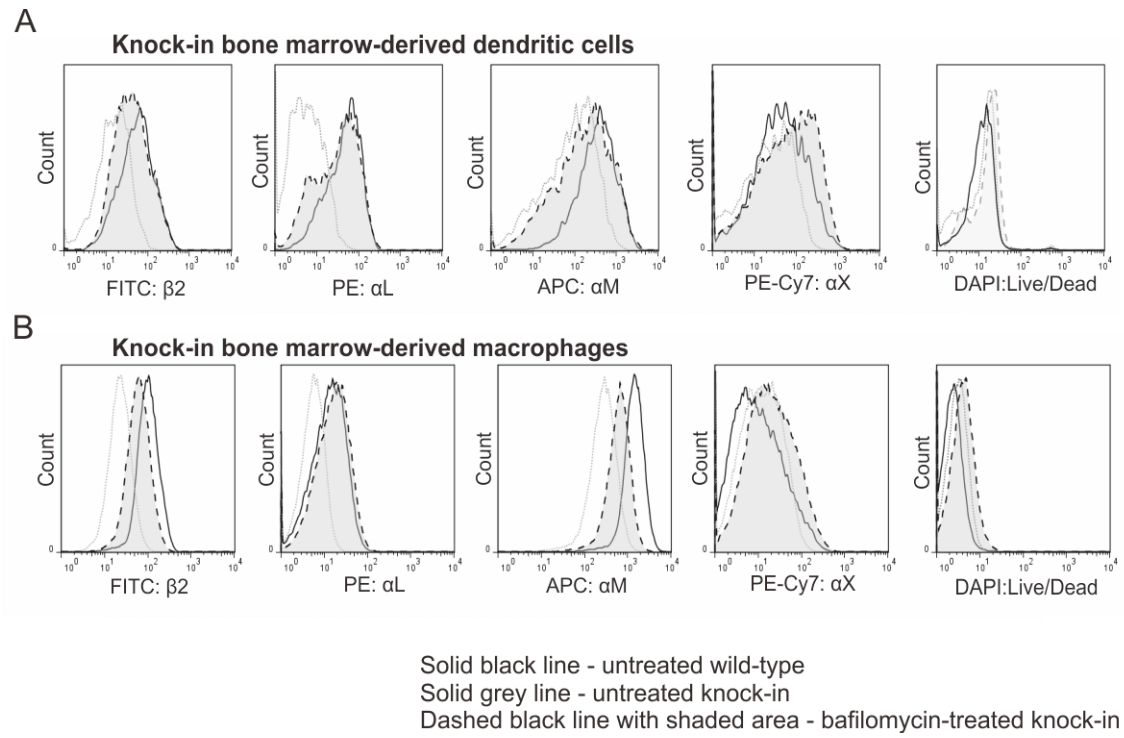


Figure 58. Bafilomycin rescued integrins from lysosomal degradation in both bone marrow-derived dendritic cells and macrophages from TTT/AAA knock-in mice.

Fluorescently-labelled antibodies were used to detect β2, αL, αM and αX integrin levels on bone marrow derived- (A) dendritic cells and (B) macrophages from wild-type and TTT/AAA knock-in mice. Solid black lines represent untreated wild-type cell levels. Solid grey lines represent untreated TTT/AAA knock-in cells levels while dashed black lines with shaded areas represent TTT/AAA knock-in cells pre-treated with bafilomycin. Cells were gated to a live population using DAPI-stain and the live/dead population is shown in the last column. Figure (58A) is also another presentation of combined data from Figure 55. Results are representative of N=3 experiments, 6 pairs of mice.

In bone marrow-derived macrophages, bafilomycin treatment increased the amount of cell surface β2, αL, αM but not αX integrins. The levels of β2 and αM integrins in bafilomycin-treated macrophages were not fully restored to the levels observed in wild-type macrophages. αL integrin levels in wild-type and TTT/AAA knock-in cells was

comparable after bafilomycin treatment but it was noted that wild-type macrophages have low levels of αL integrins (Figure 58B). Once again these results suggested that bafilomycin treatment affects the cell surface expression of LFA-1 integrins.

Unfortunately, it was difficult to determine if adhesion of TTT/AAA-knock-in bone marrow derived macrophages to ICAM-1 or iC3b ligands was affected by the mutation and/or inhibitor treatment due to the lack of suitable negative control blocking surfaces as these cells were highly adhesive to most ligands and surfaces. (Data not shown.)

5.3.4. Regulation of β2 integrin-mediated phagocytosis

5.3.4.1. The TTT/AAA mutation in the β2 integrin affects integrin-mediated phagocytosis

Since one important function of Mac-1 integrins is phagocytosis, and the TTT-motif has been previously implicated in this process in cell lines (Weidemann et al., 2006), we decided to investigate the involvement of the TTT-motif in the regulation of integrin-mediated phagocytosis. Macrophages which express high amounts of Mac-1 were chosen for phagocytosis assays. In order to quantify results, fluorescence microscopy was used to visualise the uptake of labelled complement-coated red blood cells by Mac-1 integrin mediated phagocytosis. Fluorescence microscopy was also used to observe β2-integrin localisation in the macrophages.

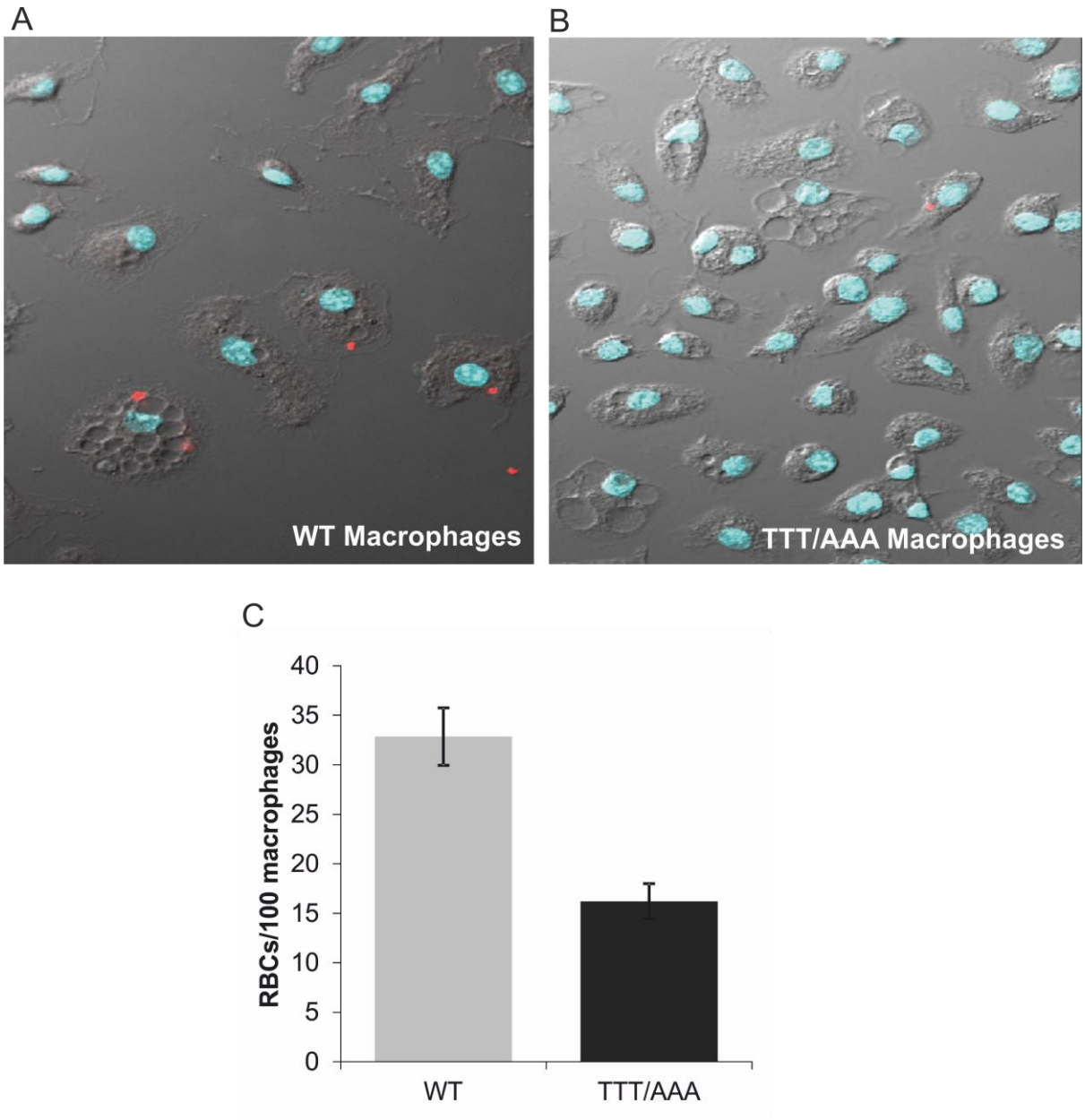
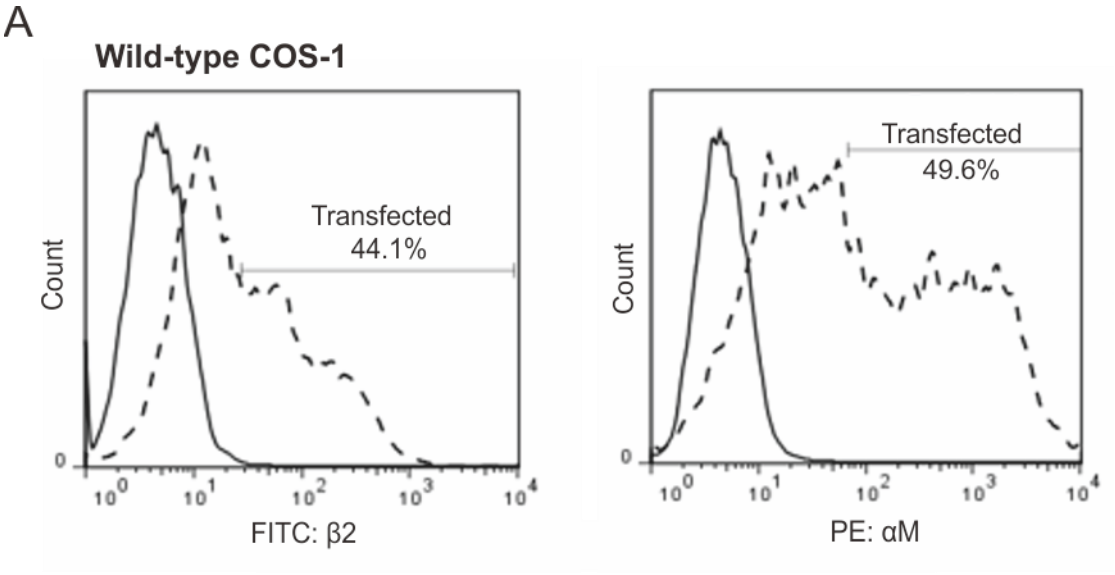


Figure 59. Bone marrow-derived macrophages from TTT/AAA knock-in mice phagocytosed significantly less red blood cells than macrophages from wild-type mice.

(A) Wild-type macrophages or (B) TTT/AAA knock-in mice macrophages were stimulated with 100 nM PMA and subjected to complement-coated red blood cells. In both cases, the nucleus of macrophages were stained with DAPI (shown in cyan) and red blood cells labelled with FITC (shown in red). (C) The number of ingested red blood cells were counted and expressed as the number of red blood cells taken up by 100 macrophages. N=2 pairs of mice, $p<0.05$ Student's t-test.

Bone marrow derived macrophages from both wild-type and TTT/AAA knock-in mice had “vacuole-like structures” after being challenged with labelled red blood cells. However, significantly fewer red blood cells were phagocytosed by TTT/AAA knock-in macrophages compared to wild-type macrophages (Figure 59). Importantly, these results confirm that the TTT-motif is important for Mac-1 mediated phagocytosis in primary macrophages, in addition to the role of this motif in regulating myeloid cell adhesion to ICAM-1 (Figure 57).

In addition to using murine bone marrow derived macrophages for phagocytosis assays, it is also possible to use COS-1 cells that can be readily transfected with WT and TTT/AAA-β2-integrins. As these cells do not contain endogenous receptors for phagocytosis, these experiments were used to confirm that Mac-1 integrin-mediated phagocytosis was affected by the mutation in the Thr758-760 site in β2 integrins. In addition, surface expression of integrins in these cells is not significantly affected by the TTT/AAA mutation (Figure 60A), in contrast to the effects seen in macrophages and dendritic cells.



C

MFI Readings

	Wild-type	TTT/AAA
Unstained	4.74	3.75
FITC: β2	29.2	20.2
PE: αM	92.2	83.1

Figure 60. Assessing the efficiency of transfection of COS-1 cells with the wild-type β2 integrin sequence or the mutated TTT/AAA sequence in the β2 integrin, together with the αM integrin.

(A) Control and transfected COS-1 cells with the β2 integrin and αM integrin were labelled with either FITC-β2 integrin antibodies or PE-αM-integrin antibodies. (B) Non-transfected and

transfected COS-1 cells with the TTT/AAA-β2 integrin and wild-type αM integrin were labelled with either FITC-β2 integrin antibodies or PE-αM-integrin antibodies. The success rates of transfection were analysed by comparison of β2 or αM integrin levels in transfected COS-1 cells (represented by dashed lines) to control non-transfected COS-1 cells (represented by solid lines). (C) Median Fluorescence Intensity (MFI) readings from the live population of cells analysed labelled with FITC-β2 integrin antibodies or PE-αM-integrin antibodies. Results shown are from one out of two experiments.

After transfecting COS-1 cells with β2 integrin and αM integrin constructs, the percentage of successfully transfected cells was analysed using flow cytometry. The population of cells with high levels of β2 integrin, which was often less than the αM integrin subunit, was determined and used for calculations in phagocytosis assays later. An example of a phagocytosis experiment after transfection is shown in Figure 62. For that experiment, the percentage of COS-1 cells that express wild-type β2 integrin was 44.1 % of the population, while COS-1 cells with mutated β2 integrins was 35.6 % (Figure 60A and 60B). Median FIourescent Intensity readouts of surface integrins showed that the levels of β2 integrins and αM integrin fluorescence between wild-type and TTT/AAA knock-in integrins on COS-1 cells were similar (Figure 60C).

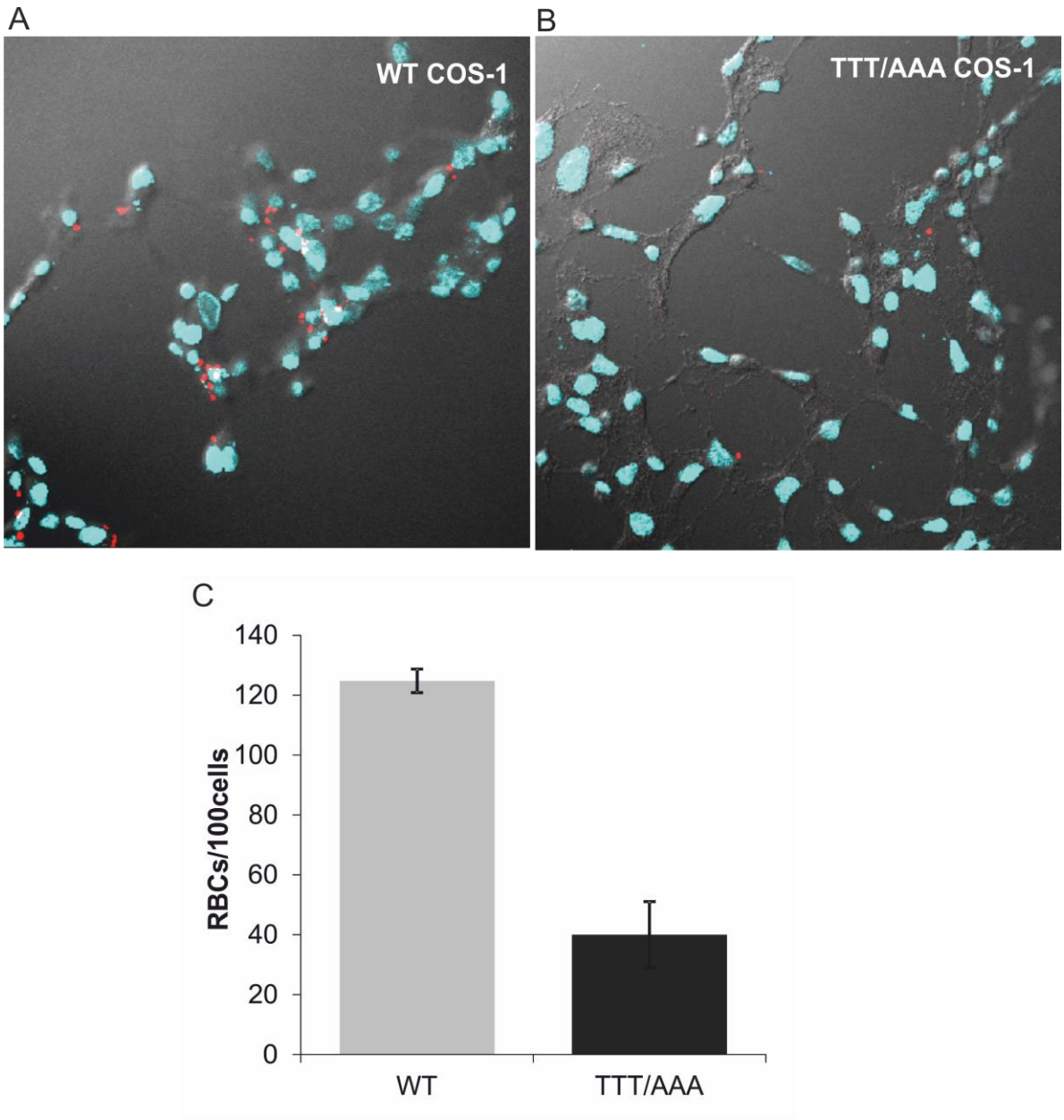


Figure 61. TTT/AAA-β2-integrin transfected COS-1 cells phagocytosed significantly less red blood cells than wt-β2-integrin transfected cells.

(A) COS-1 cells transfected with wild-type β2 integrin and αM integrin 48 hours earlier were stimulated and incubated with complement-coated red blood cells to assess phagocytosis. (B) COS-1 cells transfected with TTT/AAA-β2-integrin and αM integrin were stimulated and incubated with complement-coated red blood cells to assess phagocytosis. In both cases, the nuclei of COS-1 cells were stained with DAPI (shown in cyan) and red blood cells labelled with FITC (shown in red). (C) The number of ingested red blood cells was counted and the result is

expressed as number of red blood cells taken up by 100 COS-1 cells. N=2 sets of transfections, $p<0.05$, Student's t-test.

Transfected COS-1 cells were challenged with labelled, complement-coated red blood cells. After counting the total cells present and taking into account that only a certain percentage of cells were successfully transfected, the phagocytosis index was calculated by dividing total number of red blood cells by number of phagocytic cells (over 3-5 fields of view per sample) and multiplying it by 100, expressing it as the number of red blood cells ingested by 100 phagocytic cells. COS-1 cells that were transfected with wild-type or TTT/AAA-β2-integrins look similar before and after phagocytosis but COS-1 cells transfected with TTT/AAA β2-integrins were observed to have phagocytosed significantly less red blood cells than COS-1 cells with wild-type β2-integrins, although surface expression of integrins in these cells were similar (Figure 61).

Localisation of WT and TTT/AAA-β2 integrins during phagocytosis

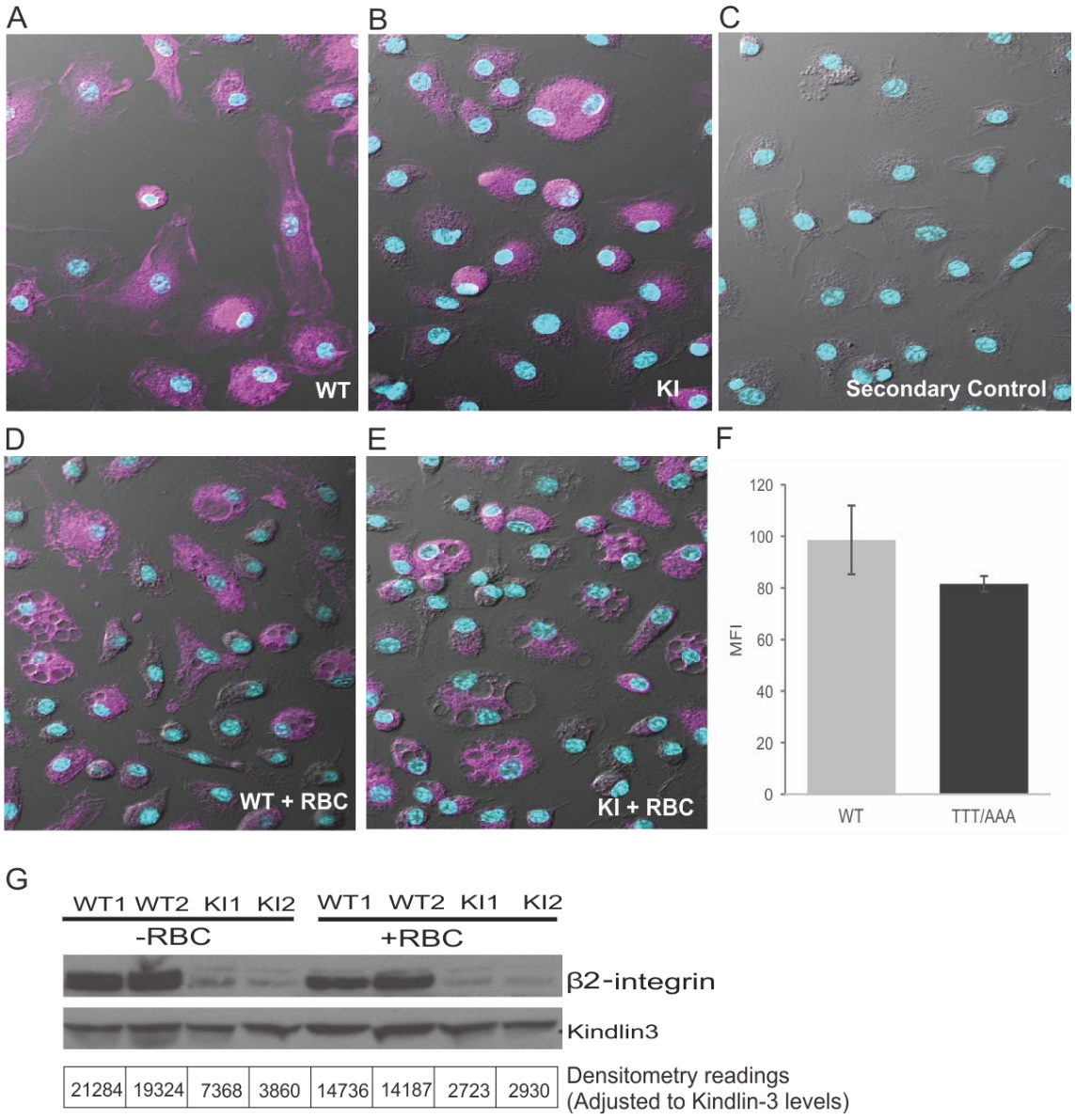


Figure 62. Localisation of β2 integrin in bone marrow-derived macrophages from wild-type and TTT/AAA knock-in mice before and after phagocytosis of red blood cells.

Detection of β2 integrins in (A) Wild-type macrophages and (B) TTT/AAA knock-in macrophages before addition of red blood cells and (C) control where macrophages were labelled with FITC secondary antibodies only but not β2 integrins. Detection of β2 integrins in (D) Wild-type macrophages and (E) TTT/AAA knock-in macrophages after addition of red blood cells (F) Macropinocytosis (FITC-Dextran-uptake) by wild-type and TTT/AAA knock-in

bone marrow-derived macrophages, which was measured by flow cytometry and expressed as MFI (Median Fluorescence Intensity). N=2 experiments. p>0.05, Student's t-test. (G) Western blot detection of β2 integrins and kindlin-3 before and after exposure of cells to complement-coated red blood cells. Western blot from cell lysates without addition of red blood cells is the same as Figure 53, while western blot from cell lysates with added red blood cells is the same as Figure 63. Representative of N=2 experiments, 4 pairs of mice.

We next investigated the localisation of β2-integrins in wild-type and TTT/AAA macrophages. In wild-type bone marrow derived macrophages, β2 integrins could be observed to form a ring at the edges of cells and β2 integrins could also been seen inside the cells. However, in TTT/AAA knock-in macrophages, β2 integrins were not observed to concentrate at the edges but were mainly found towards the centre of the cell near the nucleus (Figure 62A-62C). During the phagocytosis process, the ring of β2 integrins at cell edges could no longer be observed in wild-type macrophages. Bone marrow derived macrophages from both wild-type and TTT/AAA knock-in macrophages looked similar after being challenged with red blood cells and "vacuole-like structures" could be observed in both cell types (Figure 62D and 62E). The presence of these structures implicated that it could be possible that TTT/AAA knock-in macrophages could still be attempting to take up particles.

Macropinocytosis is a process that is not directly initiated by cell surface receptors and cargo ligands. It is result of constitutive membrane ruffling driven by active remodelling of the cortical actin cytoskeleton. Macropinosomes are also much larger and can be between 0.2μm and 5μm in diameter, compared to endocytic vesicles which are smaller than 200nm (Kerr and Teasdale, 2009, wang et al., 2010). Therefore macropinocytosis (dextran-uptake) assay was carried out using FITC-dextran to confirm that these TTT/AAA knock-in cells could still form macropinosomes. FITC-dextran is a

fluorescent substrate which could be detected using flow cytometry to measure how much has been taken up by target cells. Dextran was suggested to be used for micropinocytosis assays because it is soluble, membrane-impermeable and does not bind to cell membrane. It cannot be metabolised and does not alter cellular activities or is degraded by lysosomes. In addition, fluorescent-tagged dextran can be used in live cell imaging, visualised by light microscopy and can be used for quantification (Oliver, 1984).

Cells that were challenged were then analysed using flow cytometry and the Median Fluorescence Intensity readings were not significantly different between wild-type and TTT/AAA knock-in macrophages (Figure 62F). β2 integrins and kindlin-3 levels in the cells did not change significantly before and after exposure to complement-coated red blood cells (Figure 62G). This was not surprising as the fluorescent microscopy images of wild-type and TTT/AAA knock-in cells looked similar during phagocytosis of red blood cells.

In summary, these results suggested that TTT/AAA macrophages were able to activate non-integrin mediated macropinocytosis but Mac-1 mediated phagocytosis was impaired. This could be due to effects on both integrin recycling (in macrophages) but also due to effects on the phagocytosis process itself (binding/ingestion of particles) as the TTT-domain affects phagocytosis also in COS cells where integrin surface expression is relatively normal.

Next, we wanted to investigate which signalling pathways were activated during integrin-mediated phagocytosis of complement-coated red blood cells, and whether any differences in these processes could be detected between wild-type and TTT/AAA cells.

After the phagocytosis challenge, the cells were lysed and western blot detection of phosphorylated proteins involved in some major signalling pathways previously implicated in phagocytosis was carried out.

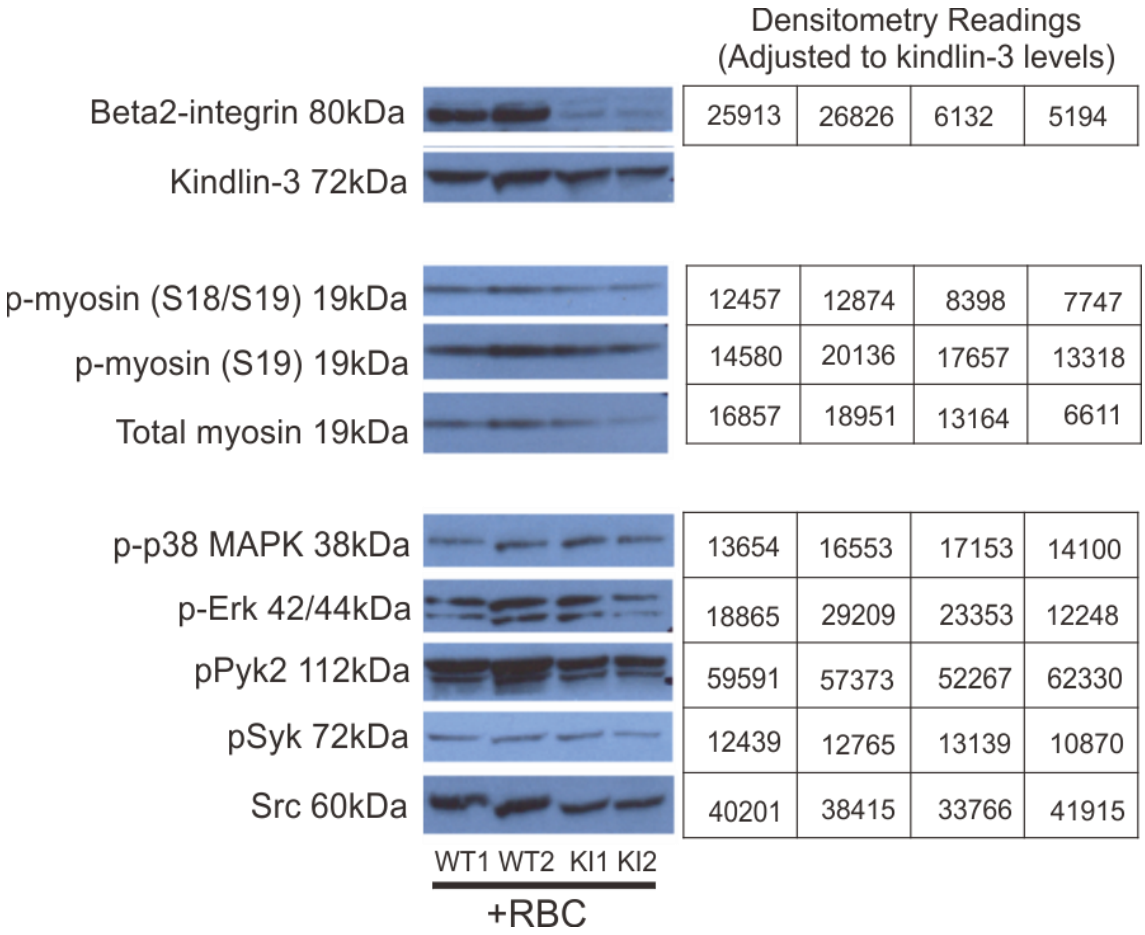


Figure 63. Investigation of possible signalling pathways involved in Mac-1 mediated phagocytosis in WT and TTT/AAA cells.

Macrophages were lysed after challenge with complement-coated red blood cells. The antibodies used for detection were β2 integrin, kindlin-3, p-myosin (Ser18/Ser19), p-myosin (Ser19), total myosin, p-p38 MAPK (Thr180/Tyr182), p-p44/42 MAPK (readout for single phosphorylation of Erk1 at Tyr204 or dual phosphorylation of Erk1 at Thr185 and Tyr187, Erk2 at Thr202 and Tyr204), p-Pyk2(Tyr02), pSyk(Tyr352) and Src. Densitometry readings were adjusted to kindlin-3 levels as controls. Results is representative of N=2 experiments, 4 pairs of mice.. Western blot detection of β2-integrin and kindlin-3 is the same blot as Figure 62.

As mentioned earlier in Figure 62G, β2 integrins levels were much lower in TTT/AAA knock in macrophages than wild-type macrophage and kindlin-3 levels were the same in all samples after exposure of macrophages to complement-coated red blood cells. Phosphorylated myosin was chosen as readout for RhoA/ROCK/myosin signalling. Active cytoskeletal reorganisation is necessary in the phagocytosis process. Rap1 has been reported to activate Mac-1 (CR3) during phagocytosis and the cytoskeletal reorganization was found to be regulated by Rho GTPases, RhoA recruitment and activation (Weidermann et al., 2006). Downstream of RhoA are ROCK and myosin which are necessary for actin cytoskeleton remodelling. We found that phosphorylated myosin light chain levels were similar in both wild-type and TTT/AAA knock-in macrophages during phagocytosis (Figure 63), indicating that the TTT-motif may not be involved in regulating this pathway in primary macrophages.

P38MAPK and ERK have been implicated in the phagocytosis process in macrophages (Kim et al., 2005). Pyk2 has also been suggested to be necessary in phagocytosis by formation of a focal adhesion complex via Mac-1 (Duong and Rodan, 2000) and tyrosine kinases such as Syk and Src was suggested to be involved in phagocytosis (Kedzierska et al., 2001, Majeed et al., 2001). Kindlin-3 binding to Mac-1 integrin has been shown to be necessary in the Mac-1/Syk/Vav-1 signalling that regulates Rac1/Cdc42 activation in cells (Xue et al., 2013). Since binding of kindlin-3 to the integrin is thought to be impaired in TTT/AAA knock-in macrophages, this could affect regulation of Syk/Vav-1. However, we could not detect differences in the phosphorylation levels of these proteins between wild-type and TTT/AAA knock-in macrophages, which suggested that other signalling factors could be affected instead (Figure 63).

5.3.5. A systemic lupus erythematosus associated variant of Mac-1 cannot mediate phagocytosis

Results from the previous sections showed the importance of the kindlin/nexin binding TTT-motif in the β2 integrin in surface expression and localisation of β2 integrins, as well as in β2 integrin mediated adhesion and phagocytosis. In humans, it has been reported that an R77H variant in the αM integrin subunit of Mac-1 was identified in patients suffering from Systemic Lupus Erythematosus (SLE) and this mutation was hypothesized to impair immune clearance thus aggravating autoimmune responses (Gaipl et al., 2005, Nath et al, 2008). Therefore, we decided to investigate the effect of the lupus-associated H77 variant on αM integrin-mediated processes.

In order to investigate the functional consequences of the R77H mutation in αM integrin-mediated processes, the T cell line Jurkat β2.7 was chosen for adhesion assays. These cells are leukocytes and do not express αL subunits but express β2 subunits, leading to no cell-surface expression of LFA-1 integrins (Weber et al., 1997). The αM subunit in the wild-type (R77) or H77 form was transfected into these cells which allows the cell surface expression of Mac-1 integrins by pairing with endogenous β2 subunits. FACS analysis of surface expression of β2 and αM were determined and they are closely similar in R77 variant and H77 variant Jurkat β2.7 cells (MacPherson et al., 2011).

Jurkat cells with H77 variant αM integrin have reduced adhesion to ICAM-1 and iC3b.

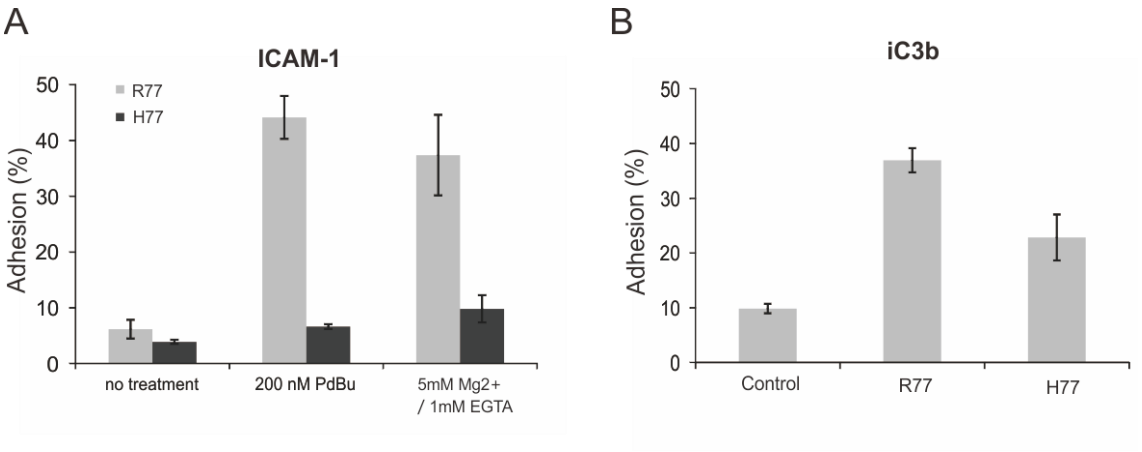


Figure 64. Assessment of the adhesion of Jurkat β2.7 cells transfected with wild-type R77 or H77 αM integrin to integrin-mediated ligands.

(A) Jurkat β2.7 cells transfected with wild-type R77 or H77 αM integrin were left to adhere to ICAM-1 after being stimulated by phorbol ester or treated with 5 mM Mg^{2+} with 1 mM EGTA. (B) Jurkat β2.7 cells transfected with wild-type R77 or H77 αM integrin were left to adhere to iC3b after being stimulated by phorbol ester. Error bars represent S.D. N=3. p<0.05, Student's t-test for all R77 compared to H77 cells when stimulated.

Mac-1 transfected Jurkat β2.7 cells that were unstimulated showed very low cell adhesion to ICAM-1. When cells were stimulated with phobol ester to activate Mac-1 integrins via inside-out signalling or when integrins were activated by use of divalent cations to bypass inside-out signalling, there was significantly higher cell adhesion of WT-Mac-1 transfected Jurkat β2.7 cells compared to cells with the H77 αM variant (Figure 64A). When cell adhesion assays were carried out using the Mac-1 ligand iC3b, untransfected Jurkat β2.7 cells showed poor adhesion to iC3b, which was expected as these cells did not have cell surface expression of β2 integrins. Cells that were transfected with the R77-αM variant had much higher adhesion to iC3b than the H77-

αM variant (Figure 64B). Taken together, these results showed that the R77 residue in the αM integrin was important in cell adhesion to ICAM-1 and iC3b and that the H77 mutation compromised cell adhesion in the Jurkat T cell line.

Jurkat cells with the H77 variant αM integrin display reduced adhesion to ICAM-1 under shear flow.

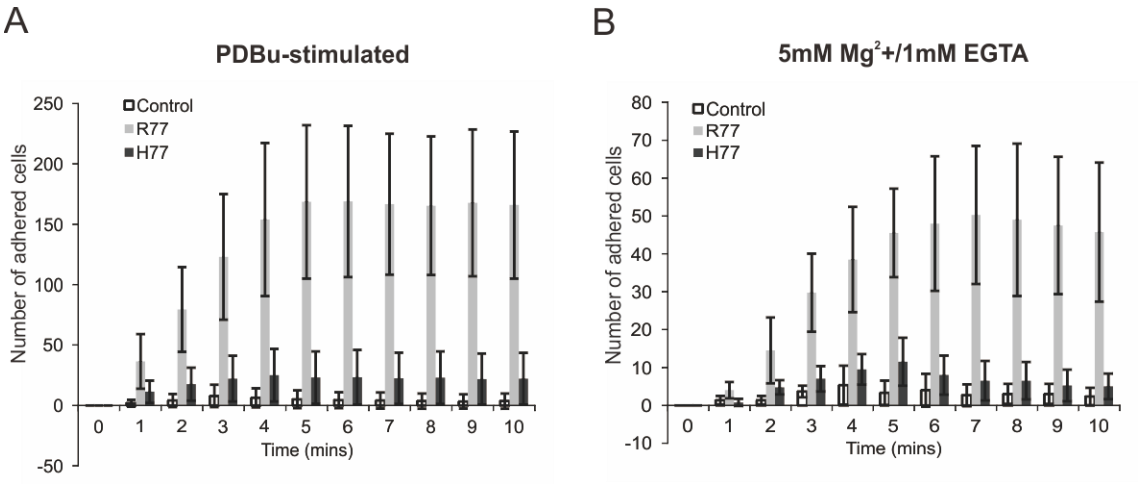


Figure 65. Assessment of the adhesion of Jurkat β2.7 cells transfected with wild-type R77 or H77 αM integrin to integrin-mediated ligands under shear flow.

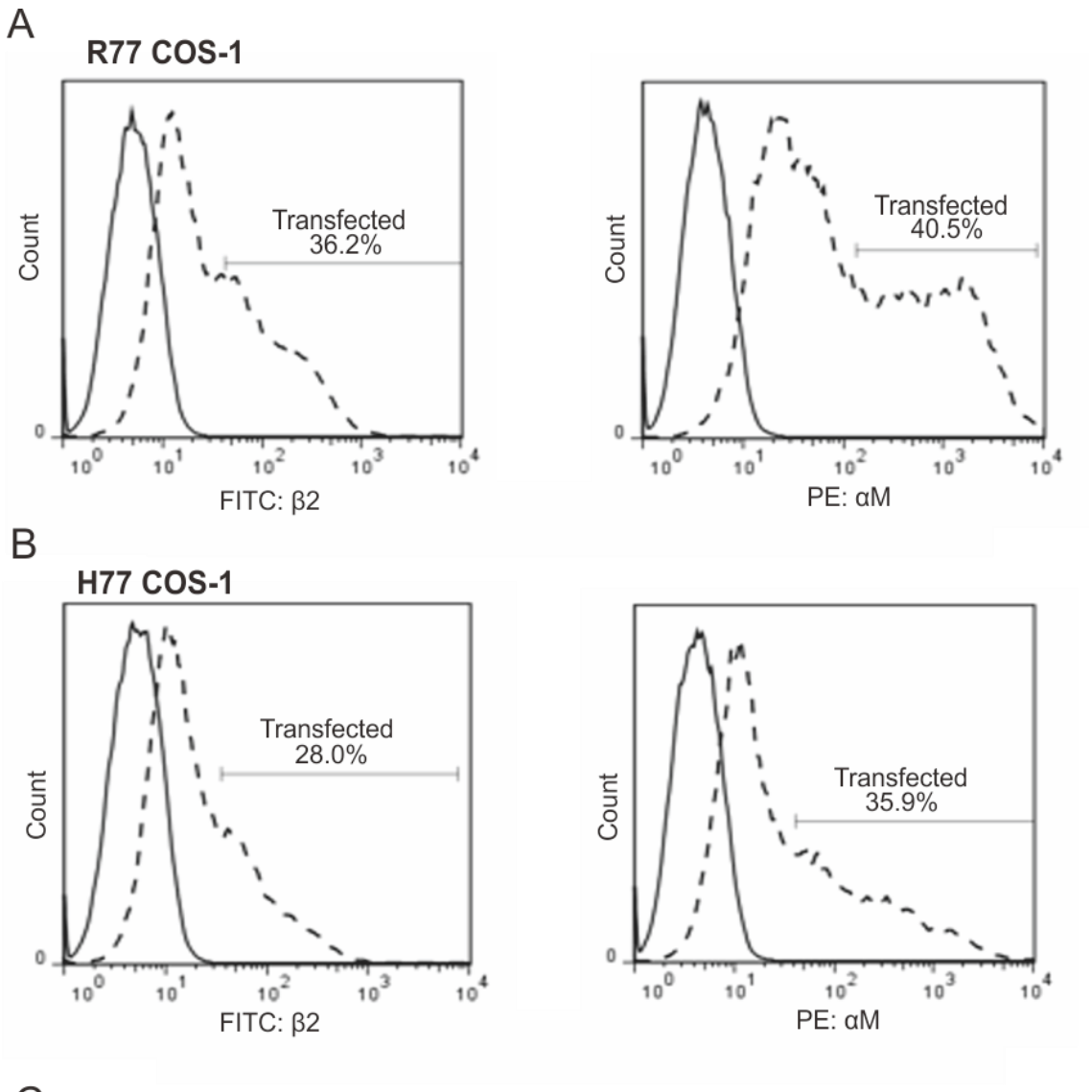
Jurkat β2.7 cells that were not transfected (control) or transfected with wild-type R77 or variant H77 αM integrin were left to adhere to ICAM-1 under shear flow of 0.3 dynes/cm^2 after being stimulated by (A) phobol ester or (B) treated with 5 mM Mg^{2+} with 1 mM EGTA. Error bars represent S.D. N=3experiments. p<0.05, (ANOVA) for all R77 compared to H77 cells when stimulated.

After assessing the adhesion of transfected Jurkat β2.7 cells in static conditions, shear flow assays were set up to compare if shear flow would further affect cell adhesion. Non-transfected Jurkat β2.7 cells were used as controls for these experiments and these

cells showed poor adhesion to ICAM-1 under shear flow despite being stimulated by phobol ester or divalent cations. Jurkat β2.7 cells that were transfected with wild-type R77-αM integrin had much better cell adhesion to ICAM-1 under flow when compared to cells transfected with the H77-αM integrins (Figure 65). These results emphasized the importance of αM in cell adhesion and suggested that the R77H mutation could cause a marked reduction in cell adhesion in both static and shear flow conditions.

The H77-variant of the αM integrin affects Mac-1-mediated phagocytosis in COS-1 cells

The results showing decreased iC3b-binding of H77-Mac-1 transfected cells led us to investigate H77-Mac-1-mediated phagocytosis. To investigate the hypothesis that the H77-αM integrins also affects Mac-1-mediated phagocytosis, H77-Mac-1 transfected COS-1 cells were used in phagocytosis assays as in the previous experiments involving Thr758-760 in the β2 integrin chain.



C

MFI Readings

	R77	H77
Unstained	5.4	5.53
FITC: β2	34.1	22.6
PE: αM	118	36.7

Figure 66. Assessing the efficiency of transfection of COS-1 cells with R77 αM integrin or H77 αM integrin, together with β2 integrin.

(A) Control and transfected COS-1 cells with R77-αM integrin and β2 integrin were labelled with either FITC-β2-integrin antibodies or PE-αM integrin antibodies. (B) Non-transfected and transfected COS-1 cells with H77-αM integrin and β2 integrin were labelled with either FITC-

β2-integrin antibodies or PE-αM integrin antibodies. The efficiency of transfection were analysed by comparing of surface β2 or αM integrin levels in transfected COS-1 cells (represented by dashed lines) to control non-transfected COS-1 cells (represented by solid lines. (C) Median Fluorescence Intensity (MFI) from the live population of cells analysed labelled with FITC-β2 integrin antibodies or PE-αM integrin antibodies. Results representative of N=3 experiments.

After transfecting COS-1 cells with β2 integrin and αM integrin constructs, the transfection efficiency was analysed using flow cytometry. An example of a transfection experiment is shown in Figure 18. The percentage of COS-1 cells that express WT-Mac-1 was about 36.2 % of the population, while COS-1 cells with mutated H77-Mac-1 was about 28.0 % (Figure 66A and 66B).

MFI readings from surface levels of β2 integrins was slightly higher in R77 variant cells than H77 variant cells while the surface levels of αM integrins was much higher in R77 variant COS-1 than H77 variant COS-1 cells. COS-1 cells transfected with R77 variant have more cells with high levels of αM integrins than H77 variant COS-1 cells (Figure 66C).

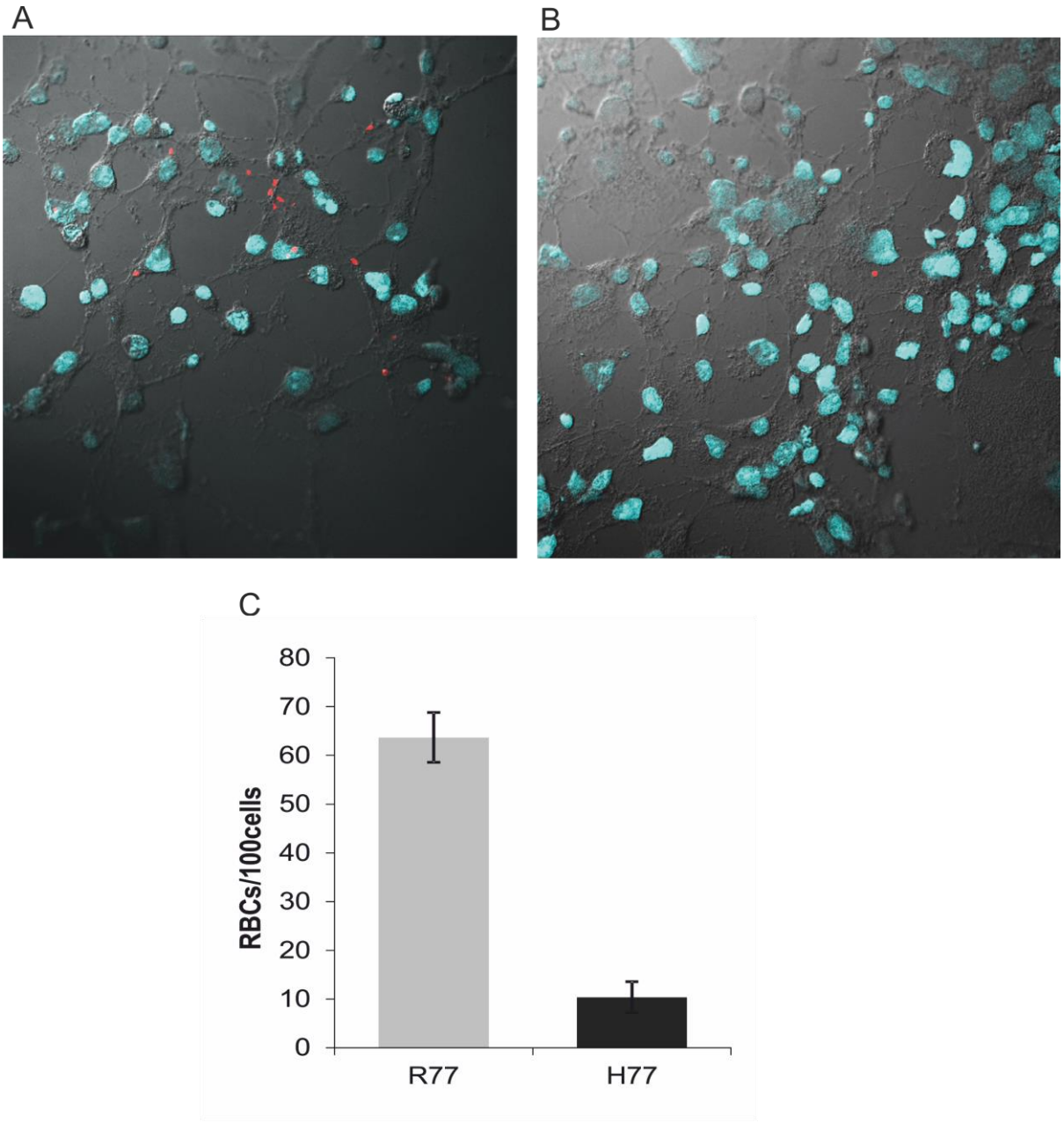


Figure 67. H77-αM transfected COS-1 cells phagocytosed significantly less red blood cells than COS-1 cells expressing R77 αM integrin.

(A) COS-1 cells transfected with wild-type β2 integrin and wild-type (R77) αM integrin were stimulated with PMA to activate integrins and then incubated with complement-coated red blood cells. (B) COS-1 cells transfected with wt β2 integrin and the H77 variant form of the αM integrin were stimulated with PMA and incubated with complement-coated red blood cells. The nuclei of COS-1 cells were stained with DAPI (shown in cyan) and red blood cells labelled with FITC (shown in red). (C) The number of ingested red blood cells was counted and the result

was expressed as number of red blood cells taken up by 100 COS-1 cells. Results are representative of N=2 experiments. p<0.05. Student's t-test.

Transfected COS-1 cells were challenged with labelled and complement-coated red blood cells. COS-1 cells that were transfected with the R77- or the H77-variant αM construct looked similar before and after phagocytosis but COS-1 cells transfected with the H77 variant were observed to phagocytose significantly fewer red blood cells than COS-1 cells with the control R77-αM construct (Figure 67). The results from the adhesion assays using Jurkat β2.7 cells and the phagocytosis assays using COS-1 cells transfected with αM variants suggested that the R77H mutation in the αM integrin affected integrin-mediated cell adhesion and Mac-1-mediated phagocytosis. These effects may contribute to SLE development in patients expressing the H77-variant of the integrin.

5.4. Discussion

Here, we have investigated the regulation of integrin recycling and phagocytosis in myeloid cells. First of all, we wanted to establish if cell surface expression of β2 integrins was affected by the TTT/AAA knock-in mutation in various cell types in the immune system. This would allow us to select suitable cell types to study β2 integrin-mediated recycling and adhesion. As phagocytosis is one of the main functions of macrophages that require integrins, we carried out assays to find out if the TTT/AAA mutation also affected this process. The second half of the chapter focused on the effects of the SLE-associated R77H-mutation in the αM integrin on cell adhesion and phagocytosis.

There was an observable difference in surface expression of β2 integrins in bone marrow derived macrophages, dendritic cells and B cells from wild-type and TTT/AAA knock-in mice. Interestingly there was no significant observable difference in levels of cell surface LFA-1 integrins between wild-type and TTT/AAA effector cytotoxic T lymphocytes (Figure 50). This could also suggest that integrins on effector cytotoxic T cells might not require nexin-17 interaction at TTT/AAA motif to prevent integrin degradation. These effector cytotoxic T cells could be regulated differently compared to bone marrow derived cells and B cells (Morrison et al., 2013).

The lower level of surface integrins in TTT/AAA-β2-integrin knock-in cells is likely due to increased degradation of integrins in lysosomes in the endocytic pathway and not due to increased proteasomal degradation, possibly due to decreased association of TTT/AAA-β2-integrins with nexin-17 (Böttcher et al., 2012).

Bone marrow-derived dendritic cells pre-treated with primaquine did now show an increased in cell surface integrins (Figure 56). Böttcher et al, 2012 showed that nexin-17 was detected from β1-integrin immunoprecipitates from cell-lysates of wild-type cells pre-treated with primaquine. Nexin-17 was not detected from β1-integrin immunoprecipitates from TT/AA knock-in cells pre-treated with primaquine. Similar experiment could be performed using β2-integrin antibodies for immunoprecipitation to examine the putative association of nexin-17 and β2-integrins in endosomes.

Using proteomics and GST-tagged pulldown assays, it has previously been shown that nexin-17 and Kindlin-2 interact with β1-integrins, but this interaction is lost when the TT-motif in β1-integrins is mutated to alanine. Nexin-17 and kindlin-2 did not interact with each other and also did not co-localise in living cells. Nexin-17 was found to co-

localise with transferrin (Tfr) and early endosomal antigen-1 (EEA1) but not with lysosomal acid membrane protein (LAMP-1) and Rab7, suggesting that the nexin-17 interaction with integrins is likely to occur in endosomes during endocytosis of integrins (Böttcher et al., 2012). Similar co-localisation studies of WT and TTT/AAA-β2-integrins using Tfr, EEA1, LAMP-1 and Rab7–tagged antibodies could be performed to observe if this happens in primary macrophages and/or dendritic cells. Nexin-17 is hypothesized to bind at the Thr758-760 site of β2 integrin in wild-type cells after kindlin-3 disassociation, which could prevent integrin degradation and thus affect integrin recycling. Using microscopy methods, co-localisation of nexin-17 with β2 integrin, or co-localisation of kindlin-3 with β2 integrin could be studied in wild-type and TTT/AAA knock-in cells or transfected COS-1 cells, with and without bafilomycin treatment.

It is still not certain when or how kindlin dissociates from β-integrin cytoplasmic tail during internalisation of integrins from cell surface (Böttcher et al., 2012, Steinberg et al., 2012). In addition, it is also unknown if integrins are still in high affinity state when internalised (Brahme et al., 2013) or if inactive integrins could also be internalised for recycling (Bouvard et al., 2013).

Kindlin-2 has been shown to compete with nexin-17 for binding to NxxY motif on β1-integrins in vitro (Böttcher et al., 2012) but it is also not known if the interaction between nexin and integrin is direct or via other adaptor proteins that bind to FERM-domains or PTB-domain. Some PTB-containing proteins that were found to bind to β2 integrins are talin, Dok-1 (signaling adaptor in cell migration) and Dab-1 (Disabled-1) (Calderwood et al., 2003). Nexin-17 also binds to PTB domains. Dok-1 is a multiple-site docking protein that mediates cell adhesion, migration and motility (Noguchi et al.,

1999, Hosooka et al., 2001). Dok-1 is a negative regulator of activation and has preference binding to β3-integrin when phosphorylated at the NPxY motif over talin, inhibiting kindlin-3 binding (Oxley et al., 2008, Bledzka et al., 2010). Dab-1 and Dab-2 binds to Amyloid Precursor Protein (APP) which also has a PTB domain and facilitates endocytosis at the cell surface. Amyloid β peptides generated by APP processing secreases are toxic in neurons and contribute to Alzheimer diseases (Hoe et al., 2006, Lee et al., 2010). Dab-1 is also a downstream target of c-Abl, which is important in regulation of neutrophil migration using β2-integrins via Vav-1 (Cui et al., 2009, Tong et al., 2012). In addition, the interaction between integrin inactivator filamin (which could bind to the FERM domains) with nexin-17 can also be investigated. In the future, it would be interesting to perform experiments for β2 integrins using available GST-tagged β2 integrin constructs (WT and TTT/AAA) to confirm if the interaction between β2 integrin with nexin-17 is direct or indirect.

The TTT-site in the β2-integrin has previously been found to be phosphorylated, which regulates the binding of 14-3-3 proteins and filamin to the integrin (Fagerholm et al., 2005, Nurmi et al., 2007, Takala et al., 2008, Gronholm et al, 2011). However, the role of β2-integrin phosphorylation in the binding of kindlin-3 or nexin-17 is unknown at present. In order to find out if phosphorylation of β2 integrin is necessary for nexin-17 or kindlin binding to the integrin, peptide-affinity chromatography experiments could be performed. Cytoplasmic peptides of the β2 integrin, in their phosphorylated and non-phosphorylated form could be coupled to agarose and used in pull-down experiments with cell lysates to investigate if nexin-17 or kindlin binding required phosphorylation at the triple threonine site, or if phosphorylation inhibits these interactions.

Integrins are glycoproteins that undergo glycosylation and heterodimer assembly in the endoplasmic reticulum and Golgi apparatus before transport to the plasma membrane. The glycosylation process regulates protein folding and reduced glycosylation of β1-integrins has been found to be involved in regulating integrin-mediated cell adhesion and migration and is involved in cancer metastasis (Gu and Taniguchi, 2008, Gu et al., 2012). Proteins that are not glycosylated bind to calnexin in the endoplasmic reticulum, leading to lower integrin export to the plasma membrane. The addition of glycosylated groups makes the integrins bulkier and more hydrophilic, which explains the band shift of mature glycosylated integrins versus their immaturely glycosylated counterparts and therefore the presence of double bands in western blot detection of β2 integrins (Morova et al., 2008 and Janik et al., 2010).

Our results show some separation of the two bands of mature and immature integrins in western blots when using β2 integrin subunit antibodies, and more immature integrins than mature integrins were detected in TTT/AAA knock-in cell lysates (Figure 53, 57 and 63). Better separation of these mature and immature integrins, especially in wild-type cells could be obtained by running the SDS-PAGE gels for longer, as shown in Figure 8B for bone marrow derived dendritic cells. The reason why more immature integrins than mature integrins were detected in TTT/AAA knock-in cell lysates is possibly that mature integrins on the surface were internalised during the recycling process but were not protected by nexins from degradation. Treatment of TTT/AAA knock-in bone marrow-derived macrophages and dendritic cells with bafilomycin resulted in increased cell surface expression of mature integrins compared to untreated knock-in bone marrow derived macrophages and dendritic cells (Figure 58). This result indicated that assembly of integrins was not affected by the mutation.

Another way to detect mature integrins on the cell surface is to label cell surface proteins with biotin and carry out immunoprecipitation of these proteins using streptavidin agarose beads, followed by detection by western blotting. Combining this method with treatment of cells with the lysosome inhibitor bafilomycin or the clathrin-dependent endocytosis inhibitor chlorpromazine in time-course experiments, it would be possible to follow the recycling of cell surface integrins over time using western blots (Maginnis et al., 2008, Vercauteren et al., 2010).

TTT/AAA knock-in bone marrow derived dendritic cells showed lower adhesion to ICAM-1 despite the rescue of cell surface integrin expression using bafilomycin (Figure 58). This is presumably due to the lack of kindlin-3 binding at the TTT/AAA site in β2 integrin which is required for integrin-mediated cell adhesion (Böttcher et al., 2012, Morrison et al, 2013). As a positive control divalent cations, such as Mg^{2+} could be used as a direct method to activate these integrins to investigate whether the TTT/AAA integrins could be functional if not for the lack of kindlin-3 binding. Divalent cations work by occupying the β I–like domain MIDAS and ADMIDAS sites to alter the conformation of integrins, thereby achieving integrin activation from the outside of the cell.

Mac-1 has an important role in facilitating phagocytosis by binding to iC3b (MacPherson et al., 2011). Phagocytosis was significantly affected by the TTT/AAA mutation in β2 integrins. Whether mutation of the kindlin/nexin binding site in the β2 integrin also affects adhesion to iC3b could be assessed using static adhesion assays in the presence and absence of bafilomycin.

Patients suffering from Leukocyte Adhesion Deficiency III have defective adhesion/signalling via integrins due to the lack of kindlin-3 or of kindlin-3 binding to the integrin (Harris et al., 2013). Patients with LAD-I have decreased neutrophils migration to sites of inflammation and it is clear that β2/Mac-1 integrins are also required for phagocytosis to clear recurrent bacterial infections (Gresham at el., 1991, Heale et al., 2001, Andrews and Sullivan 2003). However, there are limited detailed investigations on how phagocytosis by Mac-1 integrins is regulated in phagocytic cells, or how defects in integrin-mediated macrophage phagocytosis affects LAD disease. Although we have shown that the TTT-motif in β2 integrins, which is important for kindlin-3 binding to the integrin, is indeed important for cell adhesion and Mac-1 mediated phagocytosis, downstream signalling pathways that are involved in regulation in macrophages are currently unknown.

In addition, the TTT-motif is an important phosphorylation site which acts as a molecular switch in the regulation of integrin-mediated cell adhesion. When this site is unphosphorylated in resting cells, filamin, which is the negative regulator of integrin activation, is bound to this motif. During cell activation in the early stages (as shown in T cells between the first 30 seconds to 5 minutes after stimulation at the T cell receptor in T cells), talin binds to the β2-integrin cytoplasmic domain (Ghamberg et al., 2009). Kindlin-3 is then recruited to stabilize the integrin structure in the high affinity activation state, which is needed for resisting shear flow in cell adhesion and migration (Shulman et al., 2012, Morrison et al., 2013). Recruitment of 14-3-3 after phosphorylation of the TTT-motif was observed to occur later (Gahmberg et al., 2009), which in turn lead to the recruitment of Rac-1/Cdc42 which is required for cell adhesion (Nurmi et al., 2007). Whether this sequence of events occurs in myeloid cells during phagocytosis is unknown at present, and could be investigated in the future.

Signalling pathways implicated in integrin-mediated phagocytosis are for example the RhoA/ROCK/myosin pathway (Weidemann et al., 2006) and Syk/Vav/Rac1 pathways (Hall et al., 2006, Xue et al., 2013). However, initial analysis did not reveal any obvious differences in myosin or Syk phosphorylation, although further experiments under different experimental conditions should be carried out to confirm that this is the case. Vav-1 was found to be downstream of Mac-1/kindlin-3 in K562-cells as demonstrated in Xue et al., 2013 so it is uncertain if this could be observed in bone marrow-derived macrophages. On the other hand, Vav-1/3 was found to be necessary for Mac-1-mediated phagocytosis of iC3b-coated particles in macrophages, upstream of Rac; (Hall et al., 2006) this pathway could be investigated in the future in TTT/AAA-β2-integrin knock-in macrophages. This could be achieved by detection of phosphorylated and total Vav-1 levels in cell lysates in wild-type compared to TTT/AAA knock-in phagocytic cells.

Active cytoskeletal reorganisation is necessary during the phagocytosis process, and this has been suggested to be regulated by the RhoA pathway in cells (Weidermann et al., 2006). As the Thr758-760 site in β2 integrin was necessary in the regulation of Mac-1 mediated phagocytosis, studies to determine if the activation of the GTPase RhoA is affected by the TTT/AAA mutation in knock-in macrophages or dendritic cells could be carried out using pull-down kits.

Another method that could be used to investigate signalling pathway that are involved in the regulation of phagocytosis via β2 integrins is proteomics. β2 integrins and interacting proteins could be co-immunoprecipitated from wild-type or TTT/AAA knock-in bone marrow derived macrophages or dendritic cell lysates using β2 integrin

antibodies, separated by SDS-PAGE, stained and analysed by mass spectrometry to identify differences in protein complexes between WT and TTT/AAA-integrin expressing cells.

Mutations in the αM integrin are suggested to lead to hypersensitivity of the immune system, instead of the inability to respond that is associated with β2-integrin and kindlin-3 mutations in LAD. The R77H-variant form of the ITGAM gene is associated with Systemic Lupus Erythematosus, an inflammatory autoimmune disease which is characterised by defective immune clearance (Gaipl et al., 2005). The ITGAM R77H mutation is in the beta-propeller domain proximal to (near to the centre of) the ligand binding domain that mediates iC3b binding by Mac-1 integrins (Gaipl et al., 2005, Nath et al., 2008, Moser et al., 2009, MacPherson et al., 2013, Figure 49). Results here show that cells transfected with H77-Mac-1 integrins have lower adhesion to ICAM-1 and iC3b in static and shear flow conditions. The H77-mutation was found to be outside the ligand binding domain of the integrin and the integrin could still undergo activation, as detected with activation-specific antibodies (Mab24) to the integrin (Data not shown, Experiments done by Matthew MacPherson, MacPherson et al., 2011.) Therefore, the effect of the H77-mutation is not likely to affect the ability to activate integrins via inside-out signalling to bind to ligands, but may affect outside-in signalling which strengthens ligand binding. This was also supported by research done by Rhodes et al., 2012.

We have also found that the R77H mutation in Mac-1 leads to a reduction in integrin-mediated phagocytosis. Phagocytosis using macrophages derived from patient samples was also carried out recently by Rhodes et al, 2012, and the results agreed with our findings that the H77 mutation led to impaired phagocytosis. It would be interesting to

investigate whether the R77H mutation in αM integrin would directly affect RhoA-pathway or Syk/Vav/Rac pathway during the phagocytosis process using R77 and H77 COS-1 cells. This would provide a better understanding in how integrin-mediated phagocytosis in phagocytic cells is regulated to perform clearance of immune-complexes and help reduce inflammation. The H77 variant may also contribute to SLE in other ways, as inflammatory IL-6 cytokine production was observed to be higher in the H77 variant transfected U937 cells. (Experiments carried out by Matthew MacPherson, results not shown.) Other than mediating adhesion process between two cells and aiding the phagocytosis process, integrins are also signalling receptors. TNFα and IL-10 were also found to be unregulated from H77 SLE patient samples, suggesting that the adhesive properties of the wild-type Mac-1 R77 variant was able to suppress cytokine production in cells (Rhodes et al., 2012). The lack of ability to clear out deposits of immune complexes or apoptotic cells could lead to further activation of dendritic cells and macrophages and increased cytokine production (MacPherson et al., 2011). Therefore, the poor binding abilities of the H77 Mac-1 variant leads to decreased phagocytosis and signalling to regulate inflammation, and we suggest that these properties contribute to the hypersensitivity seen in Systemic Lupus Erythematosus.

Taken together, the results described here suggest that both the TTT-domain in the β2 integrin and the R77-site in αM integrin are necessary for cell adhesion and phagocytosis. With further experiments, these results may help to clarify how Mac-1 integrin-mediated phagocytosis is regulated in macrophages and the involvement of β2-integrins in disorders of the immune system.

5.5. Conclusion

The Thr758-760 site in β2 integrins is required for cell adhesion and is suggested to act through binding of kindlin-3. This site has also been suggested to be the binding site for nexin-17 which has a protective role in preventing integrin degradation in lysosomes. Treating TTT/AAA knock-in cells with the lysosome inhibitor bafilomycin led to rescue of cell-surface integrin levels to that in wild-type cells. Mutations in the Thr758-760 site in the β2 integrin affected adhesion of bone marrow derived dendritic cells to (at least) ICAM-1 ligands while the H77 variant (and not wild-type R77) in the αM integrin chain affected integrin-mediated cell adhesion to both ICAM-1 and iC3b, possibly due to defects in outside-in signalling of αM integrins. Integrin-mediated phagocytosis was regulated by Mac-1 integrins binding to iC3b, a process which required both the Thr758-760 site in the β2 integrin and the R77 site in the αM integrin. The lack of β2 integrin function affects patients with Leukocyte Adhesion Deficiency which is characterised by a weak immune response in the fight against infections while the H77 variant of the αM integrin contributes to a hypersensitive immune system observed in patients with Systemic Lupus Erythematosus. Although it is still unclear which signalling pathways downstream of Mac-1 mediated phagocytosis control these processes, these results nevertheless suggest that integrin-mediated cell processes such as phagocytosis have to be tightly regulated to maintain immune homeostasis.

6. Final conclusion and future directions

The purpose of this thesis was to investigate how β2 integrins in different leukocytes are regulated: to find out what was involved in regulating cell adhesion during inside-out signalling in B and T lymphocytes; to investigate the regulation of adhesion under shear stress conditions in B and T lymphocytes; to investigate the role of the threonine triplet (T758-760) in the β2 integrin cytoplasmic tail in adhesion and phagocytosis in myeloid cells. The effect of R77H variant of the αM-chain of the Mac-1 integrin on adhesion and phagocytosis was also studied. In this chapter, I will review the research methods and the findings from the previous three chapters and summarize the dissertation. Limitations of the work done, contribution to the current field of studies and future directions will also be discussed.

The first aim of this thesis was to investigate the involvement of protein kinases in the regulation of β2 integrins in B cells, as integrin regulation has been studied more extensively in T cells in the past. The results show that PKC isoforms may be important in phorbol ester-induced B cell adhesion but the PKCβ isoform was found not to be required for activation of integrins in B cells. It was also shown here using primary lymphocytes that the PKC downstream effector PKD does not contribute to integrin regulation in these cells, despite earlier published findings using the Jurkat T cell line (Medeiros et al., 2005).When B cells were stimulated at the B cell receptor, PI3K/Akt was shown to have an important role in integrin activation, possibly by regulating Rap1 activation. This is different to how LFA-1 in T cells is regulated, as it has been reported that PKCθ upstream of Rap1 playes a major role in LFA-1 integrin regulation (Letschka et al. 2008).

Here, we have further set up adhesion assays under shear flow conditions using SDF-1α chemokine activation of cells. This enabled the investigation of cell adhesion under shear flow, mimicking shear flow conditions in blood vessels. Earlier results from Reif et al., 2004 showed that B cells deficient in Class 1A PI3k p110δ (which is activated by chemokine-stimulated G-protein coupled receptors) and B cells pre-treated with wortmannin have reduced homing to Peyer's patches and splenic white pulp cords in adoptive transfer experiments and have reduced chemotactic response to CXCL13. Current results using shear flow assays and B cells treated with inhibitors indicated similar results as in static conditions; that some PKC isoforms and also PI3K/Akt were necessary for β2 integrin activation in B cells for firm adhesion under shear flow. However, as adhesion assays using VCAM-1 as a ligand were not carried out, it is currently unknown if VLA-4 (α4β1) integrins are affected by the same upstream protein kinases as LFA-1. Such investigations could provide more insight into how integrin-mediated migration of B cells within tissues, B cell-T cell interactions, or B cells trafficking processes in the presence of shear flow are regulated.

In addition, evidence was provided that different lymphocyte types, such as $CD4^+$ and $CD8^+$ T cells were differentially regulated upon stimulation as these cells did not depend on Akt for integrin-mediated cell adhesion in either static or shear flow conditions. The results presented here further shows that cytotoxic CD8+ effector T cells were shown to express high levels of integrins on their cell surface, could bind to ICAM-1 ligands without any cell stimulation and were observed to migrate spontaneously on ICAM-1 in static conditions. Cells that were treated with Akt inhibitors lost the ability to migrate spontaneously in 2D, indicating that Akt may be important in regulating integrin-mediated migration in conditions where shear flow is absent (e.g. in tissue). Therefore, Akt may regulate effector cytotoxic T cell function

when these cells constantly scan the environment for pathogens and kill infected cells in tissues.

A shear flow assay using effector cytotoxic T lymphocytes which were allowed to adhere to ICAM-1 only ligand, a combination of ICAM-1 and E-selectin ligands or endothelial bEnd.3 cell layers was then established. It was found that effector cytotoxic T lymphocytes, unlike B cells, did not require stimulation of inside-out signalling via chemokines for cell adhesion to ICAM-1 under shear flow to occur. These findings were in agreement with research published by Shulman et al., 2012. Using the shear flow assays, it was shown that inhibition PKC or Akt does not affect effector cytotoxic T lymphocyte β2 integrin-mediated cell adhesion to ICAM-1 under shear flow but adhesion instead requires calcium binding to calmodulin and an intact actin cytoskeleton. In addition, effector cytotoxic T cell appeared to be regulated differently in static and shear flow conditions, as inhibition of Akt in effector cytotoxic T cells had been shown to affect adhesion to ICAM-1. (Experiments carried out by Vicky Morrison.) This suggests that effector cytotoxic T cells may be able to effectively resist shear flow in the blood stream by using constitutively active LFA-1 which may aid in cell transmigration into tissues to scan for infected cells and pathogens.

Comparing the results in Chapter 3 and 4, it would appear that LFA-1-mediated adhesion in B cells is regulated similarly in the absence or presence of shear flow. In contrast, LFA-1 in effector cytotoxic T cells is differently regulated in the presence and absence of shear flow. Under shear flow conditions, adhesion is not dependt on signalling processes mediated by PKC or PI3K/Akt, but these pathways were needed for cell adhesion under static conditions, and may therefore be important for effector cytotoxic T lymphocyte migration within tissues. These results suggest that Akt may be

important for effector cytotoxic T cell functions such as migration within tissues. In the future, it could be investigated if Akt affects speed, directionality, distance travelled and displacement of effector T cells in 2D or 3D environments or in tissues.using live imaging techniques. Taken together, these results highlighted that integrin-mediated adhesion in different subsets of lymphocytes, such as B cells, $CD4^+$ cells and effector cytotoxic T lymphocytes is differently regulated, and emphasize the importance of using shear flow assays to differentiate observations related to cell-cell contact in tissues and ability to adhere during the extravasation process.

After investigating regulation of integrin-mediated adhesion in lymphocyte in chapters 3 and 4, chapter 5 focussed on the regulation of β2 integrins in myeloid cells such as dendritic cells and macrophages: particularly on the role of the T758-760 domain in the β2 integrin in regulation of integrin surface expression, adhesion and the downstream effector function phagocytosis. TTT/AAA knock-in bone marrow derived dendritic cells and macrophages have lower expression of cell surface β2 integrins than wild-type cells and these knock-in bone marrow derived dendritic cells adhered poorly to ICAM-1 when compared to wild-type cells. Cell surface integrin levels can be rescued from lysosomal degradation by treating TTT/AAA knock-in bone marrow derived dendritic cells and macrophages with bafilomycin. These results suggest that the triple threonine site was needed to prevent β2 integrins degradation in the lysosomal pathway, possibly by mediating the binding of the protective sorting nexin protein thought to be responsible for facilitating integrin recycling. Bafilomycin treatment of cells did not improve TTT/AAA-β2-integrin-mediated cell adhesion to ICAM-1, presumably because kindlin3 was still unable to bind at the mutated TTT/AAA site. These results highlighted the importance of the TTT motif by suggesting that this motif is required to maintain high levels of integrins on the cell surface and that it is also important in

regulating adhesion of β2-integrins in myeloid cells. However, the putative interactions between nexin, kindlin-3 and the β2 integrins at the TTT-motif have not yet been examined. These interactions could be investigated in the future using GST-tagged pulldowns, microscopy techniques and/or coimmunoprecipitation assays.

Macrophages with TTT/AAA knock-in β2 integrins had phagocytosed significantly less opsonised particles compared to wild-type macrophages although non-integrin–dependent macropinocytosis of dextran was unaffected. However, it is currently not known whether nexin/integrin and/or kindlin/integrin interactions are involved in regulating phagocytosis in myeloid cells. It is possible that nexin17 binds to β2 integrins at the TTT-motif (as shown for beta1-integrins in Böttcher et al, 2012), rescuing integrins from lysosomal degradation which may affects cell surface levels of integrins needed for phagocytosis. Nexin-17 knock-out or kindlin-3 knock-out mice could also be used to study if nexin-17 and kindlin-3 are important in regulation of mac-1 mediated phagocytosis. Even though it is now known that the TTT-motif in β2 integrins regulates Mac-1 phagocytosis, downstream signalling pathways that regulate macrophage phagocytosis, (such as the RhoA/ROCK/myosin pathway (Wiedemann et al., 2006) and Syk/Vav/Rac1 pathways (Xue et al., 2013)) are currently unknown and should be further investigated. However, these results may suggest that a reduction in phagocytosis may contribute to the weak ability to fight bacterial infections in patients with Leukocyte Adhesion Deficiency (Gresham et al., 1991, Svensson et al., 2000, Harris et al., 2013).

The R77H variant in the αM integrin subunit of Mac-1 has been shown to be associated with Systemic Lupus Erythematosus but the effect of the R77H mutation on Mac-1 function had not been demonstrated (Harley et al., 2008, Nath et al., 2008). We have

now shown that Jurkat β2.7 cells transfected with the H77-αM integrin chain displayed poorer adhesion to both integrin ligands ICAM-1 and iC3b when compared to cells transfected with R77-αM integrins, possibly due to defects in outside-in signalling that strengthen ligand binding. The R77H mutation was found outside the integrin binding domain. Effects of adhesion and post-receptor binding event requiring avidity of integrins for cell spreading in phagocytosis were affected the same way by this mutation on different ligands of iC3b, ICAM-1 and even plastic (MacPherson et al., 2011, Rhodes et al., 2012). Furthermore, COS-1 cells with H77-αM integrins phagocytosed significantly less opsonised red blood cells compared to macrophages with R77-αM integrin. Whether the R77H mutation in αM integrin affects the same signalling pathways as the TTT/AAA mutation in the beta2-integrin during the phagocytosis could be investigated in the future. Together, the findings in chapter 5 contribute to a better understanding of how β2 integrins can affect cell adhesion and phagocytosis in myeloid cells and the importance of these mechanisms in maintaining immune homeostasis.

What can we learn from studying β2-integrins rather than other integrin subfamilies? β2-integrins are exclusively expressed in leukocytes and are known to form transient adhesion complexes needed for leukocyte migration, which are very different from β1-integrin mediated focal adhesions in fibroblasts. Leukocytes which are rich in β2-integrins are able to migrate 100 times faster than mesenchymal and epithelial cell types (Lämmermann et al., 2009). Leukocytes can migrate at a speed of 10 μm/min and do not have focal adhesions. Transient integrin complexes are present at the front of migrating leukocytes and bigger focal zones are observed near the middle of the cell. Fibroblasts have integrin-mediated focal adhesion complexes which have a turnover rate at 10-30 minutes and are not suitable for the high migration speed of leukocytes. β2-integrins can bind to a wide range of ligands from ICAMs, plasma proteins such as

fibrinogen, fibronectin, iC3b, Factor X, extracellular matrix proteins such as collagen and laminin, and carbohydrate structures such as heparin-like glycosaminoglycans synthesized by connective tissues mast cells or released from storage granula in the inflammatory responses mediated by leukocytes. This is useful for leukocyte migration within different tissues made up of different extracellular matrix (Pinner and Sahai, 2009).

β2-integrins are also important for mediating adhesion under flow which precedes the extravasation process and this property needs to be switched on or off rapidly in the presence or absence of shear flow, although β1 integrins in the form of VLA-4 can also be utilised for cell arrest during shear flow conditions. However, the cytoplasmic tails of β2 and β1 integrins are different and potentially interact with different proteins during regulation of cell adhesion and signaling, which can also explain why different integrins are needed for different situations. We have now shown that LFA-1, one of the β2-integrins is regulated differently in B and T cells, and also in the absence or presence of shear stress. Other than mediating adhesion between cells, the β2-integrin Mac-1 is also a major integrin in regulating phagocytosis (Arnaout et al., 1983, Springer et al., 2005), which has impaired phagocytosis when mutated at the triple threonine motif at the cytoplasmic domain and in rs1143679 (R77H) lupus associated variant of ITGAM. Future examinations looking at the how LFA-1 cooperate with other integrins and adhesion molecules in different migration and effector process could contribute in understanding of how the immune system functions and how integrins could be better targeted for therapy to autoimmune diseases, atherosclerosis and cancer treatment.

In addition, β2-integrins can be easily studied using mouse models, which is especially useful for investigating abilities of lymphocyte homing, cell migration studies and

confirming cell adhesion properties in vivo as well as for other studies of β2-integrin function in the immune system in vivo. Mice with mutations in β2 integrins are viable and fertile (Marski et al., 2005, Morrison et al., 2013); this is different from mice carrying mutations in β1-integrins (Stephens et al., 1995, Czuchra et al., 2006, Meves et al., 2013).

This thesis has dissected signalling pathways which regulate integrin function in various immune cell types using primary cells from mice, rather than in cell lines. These processes could be differentially regulated in cell lines than in primary cells, as demonstrated in the study of PKD signalling. Medeiros et al., 2005 suggested that PKD possibly regulated LFA-1 integrin activation via Rap1 using Jurkat T cells but the current data using mice cells and in vivo studies showed that PKD is not important for these processes (Matthews et al., 2012). These studies thus emphasize the need to use primary cells from mice or human to study integrin-mediated cell adhesion and that, ideally, in vivo experiments should also be conducted to confirm cell homing patterns.

There are some limitations and points to consider for future work. Most of the results obtained about the involvement of AGC kinases in inside-out signalling in lymphocytes have used inhibitors which could affect other signalling pathways (e.g. could have off-target effects). Unfortunately, it was not possible to get hold of suitable transgenic mice models to confirm the effects of Akt and PKC isoforms in lymphocyte adhesion. As either the deletion lead to embryonic lethality or some of the isoforms may have overlapping functions (e.g. redundancy) which may be difficult to investigate individually. Interestingly, conflicting information on how Akt and PKC may affect each another in cell lines has been reported but this issue has not been studied in lymphocytes; this could be investigated in the future.

One important point that this thesis raised is that the regulation of lymphocyte adhesion is different in static and shear flow conditions. It is possible that the presence of shear stress alone physically “opens” up integrins for ligand binding which could over-ride inside-out signalling (Alon et al., 2007, Woolf et al., 2007). This has to be taken into consideration when assessing cell adhesion during extravasation to cross the blood/-tissue barrier. There is evidence provided by this thesis that show that shear flow adhesion assays rather than static assays have to be used to mimic processes occurring in blood vessels. For example, it is now shown that inhibition of Akt in effector cytotoxic T lymphocytes affected LFA-1 integrin-mediated cell adhesion in static conditions but Akt inhibition did not affect adhesion under shear flow.

Activation of integrins under shear flow could be investigated using antibodies such as KIM127 and mAB24 that bind to specific sites that are exposed when the human β2 integrins open up to adopt the “intermediate” or “high affinity” structure (Figure 4, Kuwano et al., 2010, Johannson and Mosher 2013). As such antibodies for mouse β2 integrins are not available, these studies would have to be carried out using human cells. It would also be interesting to study signalling pathways that are activated downstream of integrins under shear flow in the future. One way to do this could be to allow cells to first adhere to integrin ligands, and then introduce shear flow and fix the cells after a period of time, and detect the activation of signalling pathways in these cells, for example using immunofluorescence microscopy with specific phospho-specific antibodies. Cells subjected to shear flow could then be compared to cells not exposed to shear flow. Such shear flow assays could also be carried out using the Svec4-10 cell line, which is another murine endothelial cell line from axillary lymph (Siegelman et al., 2000, Ledgerwood et al., 2008). For experiments using human lymphocytes, HUVECS are commonly used (Cinamon et al., 2001, Jalali et al., 2001, Sircar et al., 2007).

The establishment of shear flow assays to study cell adhesion has also opened up possibilities in biophysics, cardiovascular and cancer studies. For example, current methods to quantify cell adhesion rely on calculating the number of cells adhering to ligands. Although Atomic Force Microscopy methods are able to directly measure the strength of integrin-ligand binding forces, this is still limited to static conditions. In the future, it may be possible to develop new methods combining optical tweezers and shear flow conditions to measure integrin binding forces in cells in shear flow. Shear flow assays could also be important in investigating pathological conditions, such as immune cells like $CD4^{+}CD28-$ T cells which accumulate in atherosclerosis (Schramm et al., 2004, Téo et al., 2004). Shear flow studies could also be a useful tool in studying how cancer cells interact with immune cells such as macrophages and how cancer cells can resist shear flow to seed in another location during metastasis.

Overall, we have successfully investigated how β2 integrins, which are exclusively expressed on leukocytes, are regulated differently by signalling pathways and interacting cytoplasmic partners in different types of primary immune cells so that these cells can migrate to specific site and perform their target functions. We have also commented on how these studies could contribute to the field of integrin studies and understanding of the immune system.

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Appendix: List of Publications

Fagerholm, S.C., Lek, H.S., and Morrison, V.L. (2014). Kindlin-3 in the immune system. Am J Clin Exp Immunol *3*, 37–42.

Lek, H.S., Morrison, V.L., Conneely, M., Campbell, P.A., McGloin, D., Kliche, S., Watts, C., Prescott, A., and Fagerholm, S.C. (2013). The Spontaneously Adhesive Leukocyte Function-associated Antigen-1 (LFA-1) Integrin in Effector T Cells Mediates Rapid Actin- and Calmodulin-dependent Adhesion Strengthening to Ligand under Shear Flow. J. Biol. Chem. *288*, 14698–14708.

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